Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium

A DISSERTATION SUBMITTED TO THE TAMIL NADU Dr.M.G.R.MEDICAL UNIVERSITY ,IN PARTIAL FULFILMENT OF REGULATIONS FOR THE AWARD OF M.D.DEGREE IN PHARMACOLOGY (BRANCH VI) EXAMINATION TO BE HELD IN APRIL 2016

Department of Pharmacology and Clinical Pharmacology

CERTIFICATE

This is to certify that this dissertation entitled “Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium” is a bona fide original work of Dr. Sumalya Sen under the guidance of Dr. Jacob Peedicayil, Professor, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, towards partial fulfillment of university regulations for the award of M.D. Pharmacology (Branch VI) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April, 2016.

Place:

Date:

PRINCIPAL

Dr. Alfred Job Daniel

Professor,

Department of Orthopaedics

Christian Medical College

Vellore – 632 004

CERTIFICATE

This is to certify that this dissertation entitled “Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium” is a bona fide original work of Dr. Sumalya Sen under the guidance of Dr. Jacob Peedicayil, Professor, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, towards partial fulfillment of university regulations for the award of M.D. Pharmacology (Branch VI) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April, 2016.

Place:

Date:

Dr. Kalpana Ernest

Professor and Head

Department of Pharmacology & Clinical Pharmacology

Christian Medical College

Vellore – 632 004

CERTIFICATE

This is to certify that this dissertation entitled “Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium” submitted by Dr. Sumalya Sen, in partial fulfillment of university regulations for the award of M.D. Pharmacology (Branch VI) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai is a bona fide original work done under my direct guidance and supervision and completed to my utmost satisfaction.

Place:

Date:

Dr. Jacob Peedicayil

Professor

Department of Pharmacology & Clinical Pharmacology

Christian Medical College

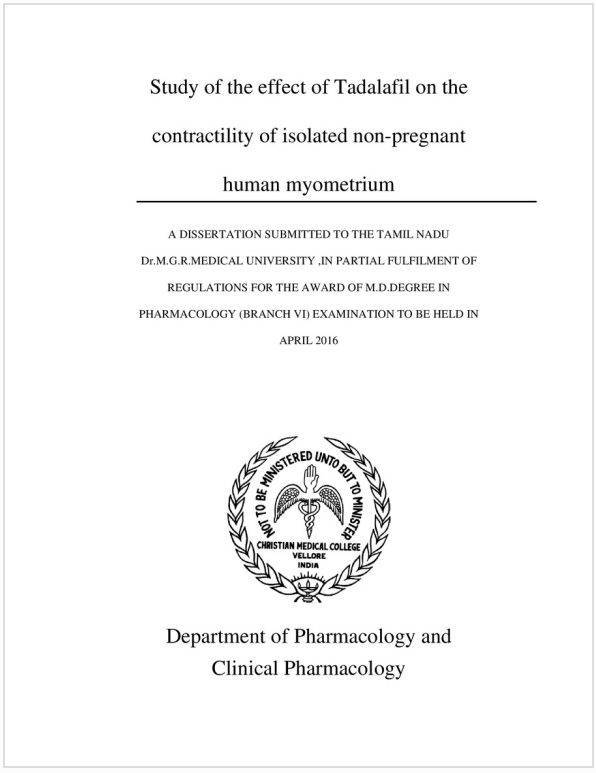
Vellore – 632 004

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	201316252.pharmacology : DR.SUM..
Assignment title:	TNMGRMU EXAMINATIONS
Submission title:	CMC_Pharmacology
File name:	Sumalya_Final_V1.pdf
File size:	1.1M
Page count:	112
Word count:	15,709
Character count:	86,800
Submission date:	22-Sep-2015 02:38AM
Submission ID:	572512920



Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium

A DISSERTATION SUBMITTED TO THE TAMIL NADU Dr.M.G.R.MEDICAL UNIVERSITY ,IN PARTIAL FULFILMENT OF REGULATIONS FOR THE AWARD OF M.D.DEGREE IN PHARMACOLOGY (BRANCH VI) EXAMINATION TO BE HELD IN APRIL 2016

Department of Pharmacology and Clinical Pharmacology

preferences

Originality Report

Processed on: 22-Sep-2015 02:42 IST
ID: 572512920
Word Count: 15709
Submitted: 2

Document Viewer

CMC_Pharmacology

By 201316252.pharmacology : DR.SUMALYA SEN

Similarity Index	Similarity by Source	
6%	Internet Sources:	3%
	Publications:	6%
	Student Papers:	1%

include quoted include bibliography excluding matches < 12 words mode: show highest matches together

Study of the effect of Tadalafil **on the** contractility **of** isolated non-pregnant **human** 15

myometrium

A DISSERTATION SUBMITTED TO THE TAMIL NADU Dr.M.G.R.MEDICAL UNIVERSITY ,IN PARTIAL FULFILMENT **OF** REGULATIONS FOR **THE** 26

AWARD OF M.D.DEGREE IN PHARMACOLOGY (BRANCH VI) EXAMINATION TO BE HELD IN APRIL 2016 Department of Pharmacology and Clinical Pharmacology CERTIFICATE

This is to certify that this dissertation entitled 4

"Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium" is a bona fide original work of Dr. Sumalya Sen under the guidance of Dr.

Jacob Peedicayil, Professor, **Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore,** 5

1 1% match (publications)
Shah, N. H., E. Thomas, R. Jose, and J. Peedicayil. "Tramadol inhibits the contractility of isolated human myometrium", Autonomic and Autacoid Pharmacology, 2013.

2 < 1% match (student papers from 19-Oct-2009)
Submitted to University of Nottingham

3 < 1% match (publications)
Tonous Silfani. "Vascular smooth muscle and exercise", Research in Sports Medicine, 1999

4 < 1% match (Internet from 02-Sep-2012)
http://27.251.28.59:8080

5 < 1% match (Internet from 11-Oct-2014)
http://www.cmch-vellore.edu

6 < 1% match (publications)
V TERZIDOU. "ENDOCRINOLOGY OF PARTURITION AND PREPARATION FOR

DECLARATION

I, Dr. Sumalya Sen, do hereby declare that this dissertation entitled "Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium" has been done by me under the direct guidance of Dr. Jacob Peedicayil, Professor, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, in partial fulfillment of university regulations for the award of M.D. degree in Pharmacology (Branch VI). I have not submitted this dissertation in any part or full to any other university or towards any other degree.

Place:

Date:

Dr. Sumalya Sen

ACKNOWLEDGEMENTS

First and foremost I thank my Lord God for the completion of this research work. He enabled my guide, co-investigators and me with necessary intellect and wisdom in carrying forward the project which may help in steering the progress of future research in this subject. Secondly I would like to thank all the patients, both cheerful and doubtful, who consented for the study without whom; this work wouldn't have been possible.

I would like to thank my parents Mr. S P Sen and Mrs. Mira Sen and my in – laws Mr. N K Mondal and Mrs. Chandana Mondal for their constant love and support at all times. I thank my wife Shreya for helping me concentrate in my work by taking care of every need of the family and also mine. I thank my beautiful daughter Sristi for the smile she brings to me, every time I see her and being generous and forgiving me for the times I don't spend with her.

I wholeheartedly thank Dr. Kalpana Ernest, Professor and Head of the Department of Pharmacology and Clinical Pharmacology for encouraging and guiding us from the beginning to the end of our MD program and for the endless support she has provided in carrying out this research work.

I express my sincere gratitude to Dr. Jacob Peedicayil, for his genuine involvement in this project. His sincerity in helping us with each and every troubles and obstacles we faced while doing this project was commendable. Above all I am truly indebted to him for the support he provided and the time

he spent with me for carefully planning and scrutinizing this project. I could not have imagined having a better advisor and mentor for my study.

Besides my guide I would like to specially thank Dr. Elsy Thomas and Dr. Abraham Peedicayil for providing tissue samples for the experiments. Also I would like to thank my other esteemed professors Dr. Manoj G. Tyagi, Dr. Denise Fleming, Dr. Binu Susan Mathew, Dr. Margaret Shanthi FX and Dr. Ratna Prabha Gupta for their insights and inspiration.

I would like to thank Aniket for helping me with skills and techniques. I would like to thank my colleague, Dr Saibal Das, for the constant support, intelligent suggestions and criticisms he made which helped me in making this project a truly memorable one. I also thank my seniors Dr. Blessed Winston and Dr. Sumith K Mathew for all the guidance with tissue handling and experiment standardization.

I would like to thank Mr. Anbarasu and Mr. Karunakaran for their help in timely and safe transportation of tissue samples and help in the lab. I thank Mr.Prabu and Mr. Subash for translating my consent form in Tamil language. I thank my other colleagues Dr.Girish Naik, Dr Rohit Kodagali, Dr Ashwathy, Dr Jaya Ranjalkar, Dr.Deepashree Sukumaran, Dr.Jayanta K Dey and Dr.Jeana for the stimulating discussions and all the good times we had.

Table of Contents

ABSTRACT

ABSTRACT

OBJECTIVES:

It is well established that tadalafil inhibits phosphodiesterase‑5 (PDE-5) leading to increased cellular levels of cGMP. Tadalafil has been shown to relax various isolated smooth muscles. However, its effect on human myometrium has not been determined. Hence, the study was conducted to determine whether tadalafil inhibits the potassium chloride (KCl)-induced contractility of isolated non-pregnant human myometrium, and if so, to study the probable mechanism of action involved.

METHODS:

Myometrial tissue was obtained from 11 patients who underwent hysterectomy. The effect of tadalafil on 55 mM KCl‑induced contractility of isolated non-pregnant human myometrium was studied using a physiograph. The ability of the specific calcium‑sensitive potassium (BKCa) channel blocker iberiotoxin (100 nM) to reverse the inhibitory effect of 40 μM tadalafil on KCl‑induced myometrial contractility was also studied. The percent inhibition caused by tadalafil with and without iberiotoxin was calculated and statistically analyzed using the Wilcoxon signed-rank test in 'R' program (3.1.1).

RESULTS:

Tadalafil produced a statistically significant inhibition of KCl‐induced myometrial contractility. The inhibition of 40 μM tadalafil on myometrial contractility was totally and significantly reversed by the concurrent administration of iberiotoxin.

CONCLUSIONS:

These results suggest that tadalafil inhibits the contractility of isolated non-pregnant human myometrium. The results also suggest that tadalafil does so by opening BKCa channels. Hence tadalafil could possibly be evaluated for use as a uterine relaxant for the management of clinical conditions like preterm labour that require myometrial relaxation.

Key words: Contractility, myometrium, tadalafil, non‐pregnant, iberiotoxin.

INTRODUCTION

INTRODUCTION

The uterus, the cavern where each and every human being is originated is a mysterious organ. It varies in structure and function considerably according to the immediate physiological needs of a female body. It follows a pristine physiological rhythm which governs the fertility of the reproductive woman and the survival and the parturition of the fetus. The complex anatomy of the uterus at various levels contributes to the above processes in harmony.

The variety of disorders of the uterus is a unique feature by itself. There are certain disorders that mainly deal with increased contractility of the uterus and certain disorders that deals with decreased contractility of the uterus. In case of decreased contractility, like prolonged labor, there is need for augmentation or induction of the labor in some cases (1). But when there is increased contractility, e.g. dysmenorrhea or preterm labor, we need to relax the uterine smooth muscle by giving certain drugs. Primary dysmenorrhea is caused by excessive uterine muscle contractions. The treatment for dysmenorrhea commonly includes nonsteroidal anti-inflammatory drugs, oral contraceptive pills and GnRH analogs (2,3). Tocolytics like glyceryl trinitrate (4), nifedipine (5) and magnesium (6) have also been tried with reassuring success for dysmenorrhea. Herbal and dietary therapy has also been tried in the ancient societies for the same (7).

The problem of preterm labor is intractable with rate of prevalence around 5-15 % in different countries including India depending on demographic and geographical features (8). Among the multiple causes of preterm delivery preterm labor is the leading cause (8). Cerebral palsy, lung disease, deafness as well as neonatal death can be the sequelae of preterm delivery. In developing countries the neonatal mortality rate is high as the care for these babies is expensive, often beyond the reach of the poor population. Even though the survival of preterm babies has improved tremendously, the cost of care, survival rate (as compared to full term) and preterm related complications have not improved much. Tocolytics causing relaxation of myometrium, are commonly used group of drugs to stop the progression of preterm labor. Even though there are multiple groups of drugs available for treatment of preterm labor, it is a dilemma to select the first line drug due to lack of specificity, presence of side-effects, and weak evidence for support of the use (9). Drugs used as tocolytics in preterm labor include β-sympathomimetic, calcium channel blocking agents, oxytocin receptor antagonist, prostaglandin synthetase inhibitors, and magnesium sulphate. All these drugs have their own advantages and disadvantages. β-sympathomimetic (ritodrine, terbutaline) can cause arrhythmia, pulmonary edema,vasculitis – as a gross their efficacy is lower than calcium channel blockers and oxytocin receptor antagonist, so they are not recommended as a tocolytic agent.

Because of less side effects and superior efficacy, calcium channel blocking agents (nifedipine) are preferred as of now. Oxytocin receptor antagonist (atosiban) though having better side effect profile compared to calcium channel blockers, is less efficacious and costlier (10). Prostaglandin synthetase inhibitors (indomethacin) have the potential to cause fetal risks. Magnesium sulphate is also not preferred as a tocolytics due to less efficacy and increased mortality of newborn (11).

Main purpose of tocolysis is to delay delivery at least by 48 hours, to allow maximum effect of antenatal glucocorticoids which is given for maturation of the fetal lung as well as transportation of the mother to a specialized center where neonatal care is available. There is no clear guideline or a drug of choice for the treatment of preterm labor (12).

Tadalafil inhibits cGMP-specific phosphodiesterase type 5, leading to increasing levels of cGMP, causing venodilation and therefore cause erection of the penis. It is also used to treat pulmonary artery hypertension since it relaxes the smooth muscle cells in the pulmonary arterial wall (13). Contrary to popular belief, tadalafil is also safe to be used in patients with previous myocardial infarction (MI) or in risk of developing MI (14) except for the absolute contraindication of taking it along with a nitrate (15).

Tadalafil has been tried out for various other conditions. It has been shown to be effective in lower urinary tract infection in benign prostatic hyperplasia patients (16). Another congener (Sildenafil) of the same group has been shown to relax human non-pregnant myometrium (17) and pregnant

myometrium (18).This action has interestingly been found to be independent of the already known phosphodiesterase inhibitory activity and is probably related to activation of large conductance potassium channels (18). This has shown to be true in other smooth muscle tissues also (19).

The expression of receptors including BKCa receptor on a pregnant human myometrium varies widely from that of the non-pregnant human myometrium (20). The action of tadalafil or its mechanism of action on non-pregnant myometrium is not determined till date.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

Hypothesis:

- Tadalafil can cause relaxation of isolated non-pregnant human myometrium in-vitro.

Aim:

- To determine whether tadalafil, a phosphodiesterase inhibitor, inhibits the contractility of isolated non-pregnant human myometrium.

Objective:

1. To study the effect of tadalafil on the height and area under the contractile curve of potassium chloride induced contraction of human non-pregnant myometrium.
2. If any relaxant effect of tadalafil is found - study the probable mechanism of action using suitable reversal agents.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Anatomy of Uterus

In the female reproductive system one of the vital organs is the uterus. It is a hollow and thick walled muscular structure. The body of the uterus extends from the fundus in the upper end to the cervix in the lower end. The uterine wall is made up of three main layers. From inside outward these layers are the endometrium, myometrium, and perimetrium. Amongst these three layers myometrium is the largest component.

The myometrium is composed of a fibromuscular layer. Smooth muscles are present in this fibromuscular layer. In this fibromuscular meshwork blood vessels, lymphatic vessels and nerves are distributed. This muscle layer is often subdivided into four more layers, from inside outward those are: longitudinal muscle layer, oblique muscle layer, circular muscle layer, vascular layer along with longitudinal muscles. Beyond this outer vascular layer with longitudinal muscles there is the perimetrium.

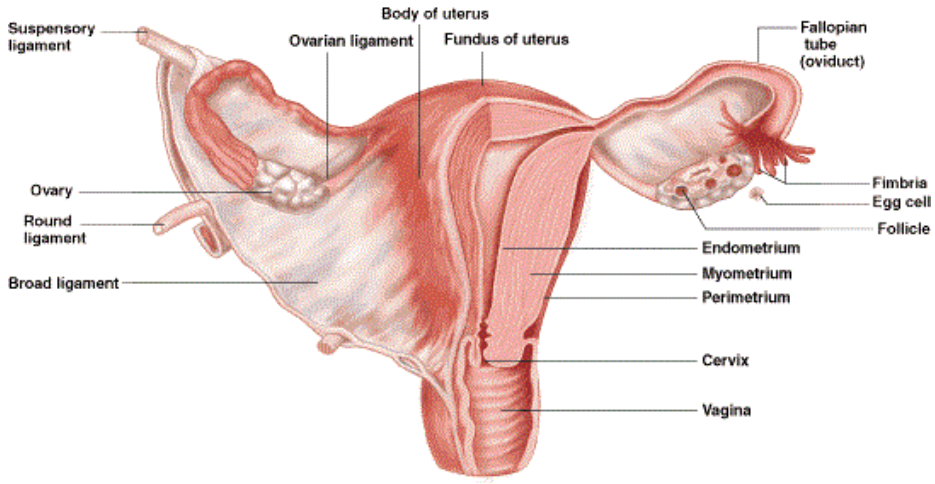


Figure 1. Anatomy of Uterus

Microstructure of the Body of Uterus

The body of the uterus comprises three layers. These are (from lumen outwards): endometrium (the mucosa), myometrium (the muscular layer) and serosa (the adventitia, part of the visceral peritoneum)

Endometrium

It is the highly vascularized mucosal layer of the uterus, velvety in appearance. It is formed by a single layered simple columnar epithelium supported by the endometrial stroma and a layer of connective tissue. The innermost columnar cell layer is ciliated and secretory and lines the lumen of the uterus. The underlying stroma is a thick layer of lamina propria (areolar connective tissue). Endometrial glands invaginate from the columnar epithelium to the myometrial layer. The endometrium is functionally divided into two layers— stratum functionalis, which lines the lumen and sloughs off periodically with menstruation and stratum basalis, which regenerates the stratum functionalis before the next menstruation. The arcuate arteries arranged circularly in the myometrium branch and penetrate deep as radial arteries and divide into two kinds of arterioles— straight arterioles which supply the stratum basalis and spiral arterioles which supply the stratum functionalis. Spiral arterioles change significantly with the menstrual cycle. The extensive vasculature of this layer helps in the implantation of the embryo and development of the placenta.

Myometrium

The exact orientation of the myometrial fibers remain controversial (21), yet traditionally it is believed to consist of four muscular layers (22):

a) Submucosal layer (innermost) — mainly longitudinal and some oblique fibers, except at the os where it forms a circular ring
b) Vascular layer— thick layer with longitudinal fibers and vessels
c) Supravascular layer— thick layer with predominantly circular fibers
d) Subserosal layer— outer, thin layer with longitudinal fibers

The muscular fibers of the outer two layers of the uterus converge at the isthmus of the fallopian tube and continue in it with some fibers reaching the uterine ligaments. The muscle fibers merge with the connective tissue containing collagen and elastin at the junction of the body of uterus and cervix.

The submucosal fibers of the lateral wall of the uterine lumen which run from the fundus to the cervix are structurally different from the rest of the myometrial fibers and may be involved in the coordination of the contraction of the uterus (22). Microscopically, muscle fibers are derived from multiple fasciculi which consist of numerous myocytes and connective tissue. Further, myocytes are arranged into bundles, which are again of multiple types

including connecting bridges formed by dense bundle of myocytes. The bundles merge, dichotomize and intertwine with each other (23). There is sufficient amount of interlacing of the uterine fibers and vast amount of heterogeneity of fibers in any given part of myometrium (along with the presence of oblique fibers) and there is no significant difference in contractility of uterine strips in-vitro when measured from transverse strips and longitudinal strips of uterus (24).

Serosa

It is composed of visceral peritoneum. There are no smooth muscle fibers in the serosa. The effect of serosa on the contractility of myometrium has not been studied before.

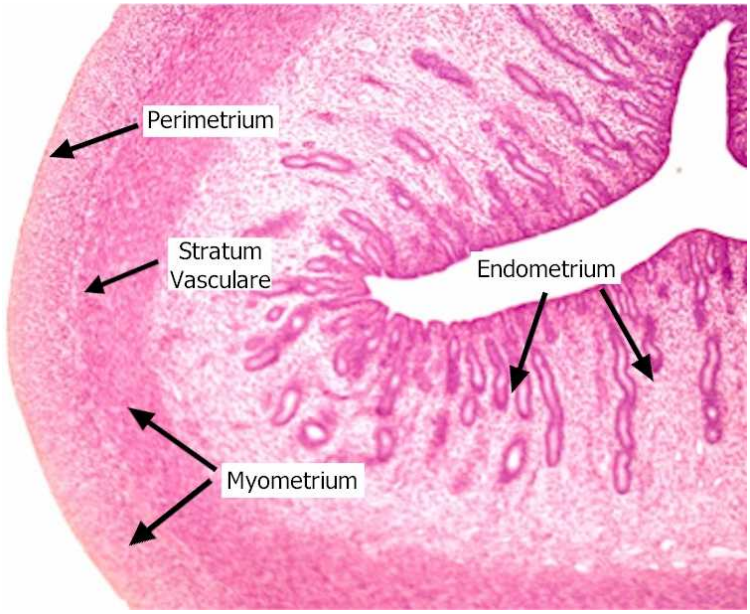


Figure 2. Layers of uterus

Vascular supply to the Uterus

Uterine arteries supply the uterus. It is a branch of the anterior division of the internal iliac artery. It reaches the uterus at the level of the cervico-uterine junction. It branches into multiple small arteries anastomosing with the ovarian artery superiorly and the vaginal artery inferiorly. The uterine artery undergoes marked hypertrophy during pregnancy. The multiple branches of the uterine artery enter the uterine wall, divide and run circumferentially as the anterior and posterior arcuate arteries. They are ramified and thinned out as they approach the anterior midline and the posterior midline, which makes the uterus less vascular in the midline. Helical arterioles are the tortuous terminal branches of the uterine artery. They continue as dense capillary plexuses in the endometrial and the myometrial layer (22).

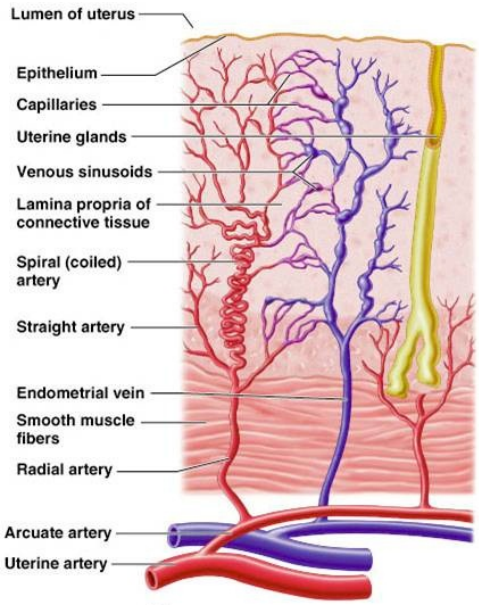


Figure 3.Vascular supply of uterine layers

The uterine veins run adjacent to the arteries and drain into the internal iliac veins. The lymphatic drainage of the uterus is into three groups of lymph nodes: the external iliac, internal iliac and the obturator group of lymph nodes. There is some amount of drainage into the para-aortic and inguinal group of lymph nodes also.

Innervation

The inferior hypogastric plexus innervates the uterus. The nerves usually accompany the vessels and end in the endometrium. Efferent pregangionic sympathetic fibers are derived from T12 to L1 spinal segments and preganglionic parasympathetic from the S2 to S4 spinal segments. Sympathetic stimulation causes contraction of the uterus and vasoconstriction while parasympathetic stimulation causes the reverse (22).

Physiology of the Uterus

The knowledge of the functioning of the normal human myometrium in various physiological states is very important for understanding the cellular mechanism of the drug under study. The uterus is a hollow muscular organ: the smooth muscles present here are capable of contracting by themselves. Upper uterine segment contraction is different from that of the lower uterine segment in the presence of prostaglandins pointing towards the heterogeneity of receptors and signaling mechanisms in different anatomical

parts of the uterus (25). Different types of modulators of contraction and excitation-contraction coupling have been studied at cellular and molecular levels in the recent past (26). The endometrium is made up of a number of different cell types and is an active, secretory structure in nature which releases a number of substances that can affect the activity of the smooth muscle layer. These substances include prostanoids, endothelin and platelet activating factor (27).

Mechanism of smooth muscle contraction

Smooth muscle cells are small cells with about 80-90% of the cell filled with myofilaments. Smooth muscle is composed of actin and myosin filament like skeletal muscle but it does not have troponin complex that is required for skeletal muscle contraction. Generally skeletal muscle have more myosin than actin, which is the opposite in smooth muscle in which there are two to ten times more actin than myosin (28). In myometrium actin is present six times more than myosin (29). So in case of smooth muscle contraction the mechanism is different compared to skeletal muscle. One of the major factors that play a role in smooth muscle contraction is change in calcium ion concentration inside the cell. Adenosine triphosphate (ATP) is broken down to adenosine diphosphate (ADP) to yield energy for contraction. Only one molecule of ATP is required for each cycle of contraction. Smooth muscle contraction is prolonged and tonic contraction.

Smooth muscle contains a large amount of the regulatory protein calmodulin. This is different in contrast to skeletal muscle contraction where troponin is required. Calmodulin activates the myosin cross-bridges. Activation and subsequent contraction occurs by the following steps:

- Calcium and calmodulin join together and form a complex.
- This calcium-calmodulin complex joins with myosin kinase and activates it (this causes phosphorylation).
- There is one regulatory chain in the myosin head. This chain get phosphorylated by the myosin kinase and it causes attachment-detachment cycling of the myosin head with actin filament leading to muscle contraction.

An increase in the calcium ion concentration is the initiating agent for smooth muscle contraction. This increase in concentration can be caused by different reasons: nerve stimulation, hormonal stimulation, change in chemical environment and stretching of muscle fiber. The diversity for this initiation is mainly due to the presence of multiple types of receptors on the surface of the smooth muscle cell membrane.

This contractile process starts reversing when the calcium ion concentration falls below a critical level. But the myosin head still remains in the phosphorylated form. Reversal of the myosin head to its normal form requires a myosin phosphatase that leads to stoppage of the cycling and cessation of contraction.

Inhibition of contraction also occurs when there is closure of sodium or calcium ions into the cells, or opening of normally closed potassium channels that leads to more positivity outside the cell leading to hyperpolarization that strongly inhibits muscle contraction. Other receptor mechanisms that causes inhibition of contraction are activation of the enzyme adenylate cyclase or guanylate cyclase. This enzyme leads to formation of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), known as second messengers. The cAMP or cGMP leads to change in the degree of phosphorylation of several enzymes that indirectly inhibit contraction.

Regulatory mechanisms

The regulation of the contractility of smooth muscles is mainly by two enzymes: calcium-calmodulin-dependent protein kinase II (contributed by Rac/CDC/42-associated kinase) and Myosin light chain phosphatase. The former reduces the affinity of calcium-calmodulin complex to myosin light chain kinase while the latter removes the phosphate from phosphorylated myosin. Myosin light chain phosphatase is regulated positively by cGMP and negatively by G-protein rho-A-rho-associated kinase and protein kinase C (PKC) (30).

Electrical properties of the myometrium

The cyclical depolarization and repolarization causes the sequence of contraction and relaxation of the uterus. Intermittent bursts of spike-like action potential contribute to the pacemaker activity of the myometrium (31). It is unclear whether there are specialized cells present to cause this spontaneous activity like that of the gut or in the urethra (32). A contraction can be initiated by a single spike, but a strong, sustained contraction requires a train of coordinated spikes. The action potential depends on the membrane ionic permeability which is both voltage-dependent and time-dependent. The frequency of the contraction depends on the frequency of action potentials within a burst, amplitude of the duration of the burst, and the duration of the number of simultaneously active cells (31).

Role of sarcoplasmic reticulum

Myometrial cells have sarcoplasmic reticulum both towards the center and the periphery of the cell and these possibly have different roles. SERCA 2 a calcium-ATPase helps sarcoplasmic reticulum store calcium against a concentration gradient. Ca2+ can be released from the sarcoplasmic reticulum by two mechanisms. One is by Ca2+ itself (CICR-Ca2+ induced Ca2+ release) and the other is mediated by inosine triphosphate (IP3)(IICR-IP3 induced Ca2+ Release). Both IP3 and the ryanodine receptor(RyR) are distributed evenly over the reticular membrane and probably use second messengers to release Ca2+ (33). RyR are calcium-release channels on which calcium acts to increase its release from the sarcoplasmic reticulum. IP3 is

present in minimal quantities in the cell and can be increased in quantity with the help of certain agonists through hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP2). There are three isoforms each of IP3 and RyR present in the myometrium. All three forms of both the receptors are present in the human myometrium. The predominant RyR form is RyR3 and its expression seems to be down-regulated towards the end of gestation. All three isoforms of IP3 receptors have been found to be present in human myometrium and their expression does not change throughout pregnancy.

Intracellular levels of calcium have to be reduced for relaxation of the myometrial cell. Calcium is extruded out of the cell by plasma membrane Ca2+ ATPase (PMCA) and a Na-Ca exchanger (NCX). These mechanisms are more important than SERCA 2-dependent Ca2+ intake into the sarcoplasmic reticulum. Level of PMCA has been shown to increase with progressing pregnancy. Blocking of CICR by ryanodine has been shown not to reduce the spontaneous contraction or contractility of the cell in rats. Oxytocin causes contraction of cells by multiple mechanisms: decreasing Ca2+ efflux, increasing Ca2+ influx and through IICR. Much about the role of sarcoplasmic reticulum in uterine contractility is under study and not clearly understood at present.

Contractant Pathways

The major receptors involved in contraction of the myometrial cell are tyrosine kinase receptor (TKR) and G-Protein coupled receptors (GPCR). Both these receptors activate phospholipase C (PLC). Oxytocin and

prostaglandins act via GPCRs. PLCβ1-4 subfamily gets activated by Gαq/II subfamily, while PLCβ2 and PLCβ3 gets activated by Gβγ released from the Gαi complex and PLCδ gets activated by Gαh. PLC helps in the production of diacylglycerol (DAG) and IP3. IP3 causes release of Ca2+ from sarcoplasmic reticulum, which is important in sustained contractility. MAPK (mitogen-activated protein kinase also called ERK - extracellular-signal-regulated kinase) pathway also can be activated by Gβγ activation. This is related to hormones related to contraction through GPCR and regulation of thin filaments. GPCR also can activate the phospholipase D pathway.

Oxytocin

Oxytocin GPCRs (OXTRs) predominantly act through the Gαq subfamily, although it can act through the Gαi and Gαh to stimulate PLCδ. The DAG and IP3 thus formed can stimulate contraction. Oxytocin can activate ryanodine receptors through TNFα-enhanced cyclic ADP-ribose (cADPR) pathway. Oxytocin acts through downstream pathways too. It can act through ERK1/2 phosphorylation suggesting involvement of the GαiGβγ-mediated pathway. There are multiple minor pathways by which ERK is activated. ERK activation plays multiple roles: contraction of the smooth muscle cell, stimulation of COX-2 enzyme and stimulation of PGF synthase. Oxytocin also induces increase in total protein synthesis and stimulates dephosphorylation of eukaryotic elongation factor 2 in the myometrium.

Prostaglandin F2α

PGF2α acts on the prostaglandin F receptor (FP) and the thromboxane receptor (TP). These receptors are present in both the nucleus and the plasma membrane. The action of PGF2α depends on the concentration of extracellular calcium as it acts through the influx and efflux mechanisms on the cell membrane.

Epidermal Growth Factor

EGF acts on the epidermal growth factor receptor which stimulates a tyrosine kinase pathway and stimulate PLCγ activity and thereby increases IP3 resulting in intracellular calcium release. It also has the conventional Ras pathway activating ERK. Its action has been associated with oxytocin receptor signaling too.

Relaxant Pathways

Relaxation of smooth muscles usually involve cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP). GPCR activate adenylate cyclase to convert ATP to cAMP, while nitric oxide activates guanylate cyclase to convert GTP to cGMP. They activate their respective protein kinases that phosphorylate proteins regulating Ca2+ homeostasis. The relaxant effect of β-blockers and prostaglandin E on myometrium is through their cAMP action (34). cAMP and cGMP concentrations are both regulated positively by respective cyclases and negatively by their phosphodiesterase, which degrades them.

Cyclic adenosine monophosphate (cAMP)

cAMP, a cyclic nucleotide is a second messenger involved in a variety of biological processes like secretory activity, learning, modulation of conductance of ion channels, production of various transmitters and messengers. In the myometrium it causes relaxation by activation of cAMP-dependent protein kinase (PKA) which in turn phosphorylates MLCK decreasing its affinity for the calcium calmodulin complex. It is formed from ATP by the action of the enzyme adenylate cyclase located on the inner side of the cell membrane. Adenylate cyclases are activated by certain GPCRs which have a wide range of agonists. The action of cAMP is stopped by its hydrolysis by certain phosphodiesterase. PDE4 (of the PDE 1—5 family found in myometrium) is the most abundant phosphodiesterase in the myometrium and its levels are significantly decreased during pregnancy maintaining the quiescence of uterus. B2-adrenergic agonist also acts through cAMP to cause uterine relaxation.

Cyclic guanosine monophosphate (cGMP)

Cyclic GMP is formed by the enzyme guanylate cyclase. Guanylate cyclase exists in two forms: soluble and particulate. Soluble guanylate cyclase is completely intracellular while the membrane bound form (particulate) consists of a extracellular domain and two intracellular domains in which one domain resembles the soluble guanylate cyclase. Nitric oxide activates soluble guanylate cyclase to produce cGMP which activates protein kinase G (PKG) which phosphorylates multiple targets. It also activates

myosin phosphatase decreasing the rate of myosin phosphorylation. There is crosstalk between the cGMP pathway and the cAMP pathway. Cyclic GMP binds to PDE2 and increases the hydrolysis of cAMP (35).

Ion channels involved in contraction

L-type voltage-dependent Ca2+ channels

These channels have a major role in sustaining contraction. The alpha subunit consists of the channel pore and drug binding sites along with the voltage sensor while the rest of the channel (alpha2, delta, gamma) subunits modulate the activity of the channel (36). The concentration of this channel varies with different layers of muscle and also with duration of gestation (37). Blockers of this channel reduce both spontaneous and induced contractions (38). Gβγ, PI-3K and PKC of the GPCR pathway can stimulate L-type channels. Oxytocin opens Ca2+-activated chloride channels, which indirectly stimulate these channels (39).

T-type Ca2+ channels

These are usually associated with spontaneous contractile activity in smooth muscles. They are also involved in calcium oscillations (Ca2+ oscillations are transient elevations of intracellular calcium followed by rapid decay, so that it can act as an intracellular signal. Also it helps in the survival of the cell since prolonged elevated calcium concentrations can be lethal to the cell). T-

type Ca2+ channels have been found to be present in human myometrium too (40). Inhibition of spontaneous contraction and oxytocin induced myometrial contractions by T-type Ca2+ channel blockers have been shown in the caprine (goat) myometrium previously (41).

Transient receptor potential channels

These channels can cause an entry of a non-selective cation or calcium when activated. They can respond to hypoxia and stretch along with hormonal signals in smooth muscle cells (42). They are reported to modulate cell migration, differentiation and growth, depolarization and tone of the smooth muscle. The transient receptor potential channel consists of multiple subfamilies: TRPM, TRPC, TRPV, TRPA, TRPP, TRPML, and TRPN. These channels establish the importance of other cations involved in the contraction of human myometrium.

Potassium channels

There are multiple potassium channels involved in the contractility of the uterus. These include the ATP-sensitive, voltage-activated and calcium activated potassium channels along with other newer channels. Potassium ions are involved mainly in reducing the excitability of the myometrium by an outward K+ current causing hyperpolarization or repolarization. The potassium channel is made of a six-transmembrane domain molecule. The six

domains S1—S6 form a pore-forming hairpin loop. Four such units join together to form the channel. Membrane voltage is detected by the S-4 domain. There is a unique signature of eight amino acids (TXXTXGYG) present within the pore of all the potassium channels identified till date (43,44).

Calcium -sensitive potassium (BKCa)

These are a collection of three different channels namely the small conductance potassium channels, intermediate conductance potassium channels and the large conductance potassium channels (denoted BKCa, Maxi K). In this the BKCa is widely distributed throughout the body and has widely been studied in the human myometrium. BKCa is also the predominant channel present in the human myometrium both in the pregnant and the non-pregnant state. It is found that in different stages of pregnancy and towards labor the function and expression of these channels are different. When these channels are sensitive to calcium it is termed as BKCa channel but when they don't have any calcium or voltage sensitivity they are termed as BK-channel (45).

These classes of channels are both voltage-dependent and calcium-dependent with a conductance of around 150–200 pS (46). These channels are blocked by tetraethylammonium, 4-aminopyridine and more selectively by iberiotoxin. The structure of the channel resembles that of the voltage

gated potassium channels except for the addition of the S7 – S10 domains. S1-S6 region have been attributed to the voltage dependence and the carboxy terminus to the calcium dependence. The BKCa channel has a α and a β subunit and their heterologous expression together is thought to increase the sensitivity for calcium (47). The quiescence of the human myometrium during pregnancy is maintained by the BKCa channel by its opposing potassium currents. During labor BKCa loses sensitivity to both voltage stimulation and increased intracellular calcium which helps in parturition (45).

*Voltage-gated potassium channel*s

There is very little known about this channel and its significance in the human myometrium. There are multiple types- rapidly acting, slow acting and the transiently acting potassium channels present in the human myometrium. There are more types of potassium currents in the pregnant myometrium (48) which necessitates further study on these channels. In a mouse model it has been shown that voltage-gated potassium (Kv) channels have regulatory role in uterine contractility (49). In vascular smooth muscle the role of Kv channels has also been proven (50).

ATP-sensitive potassium channels

These channels are unique in that they respond to metabolic activities of the cell. They close in response to increased intracellular ATP levels. They are a subtype of inward rectifier potassium channels. Their role in smooth muscles is unclear although their roles in vascular, cerebral and visceral organs are well studied. The classical example of their function is in pancreas where they cause insulin release. They have got two subunits: the pore forming channel and the sulphonylurea receptor. ATP acts on the pore forming channel subunit while the antidiabetic drug sulfonylureas act on the sulphonylurea receptor subunit. There is some electrophysiological evidence of their presence in the myometrium(51) but their role in contraction in view of their metabolic functions is yet to be studies.

Sodium and chloride channels

Both these channels have been studied in rat myocytes (52). Sodium channels cause a fast inward current depolarizing the cell membrane. Nav2.1 and Nav 2.3 are present in human myometrium and their density increases with pregnancy. Calcium dependent chloride channels are activated by increase in intracellular calcium. It results in an outward current depolarizing the myometrium. They may have a role in the spontaneous contraction of the uterus.

Pharmacology of drugs used in the study

Potassium Chloride

KCl is considered an universal agonist in contracting smooth muscle cells (53). It has been used as the agonist drug on myometrium in the past(54). The dose to be used has been standardized in our department previously (55). KCl depolarizes the cell membrane by offsetting the K+ equilibrium potential above the resting level. It is a classical case of an electromechanical coupling rather than a pharmacomechanical coupling. Unlike pharmacomechanical coupling, K+ will not contract a tissue in a calcium free solution (56). Since the final mechanism of action in both kinds of coupling is through opening of membrane calcium channels with an increase in intracellular calcium, they produce similar kind of dose-related increase in contractile activity. However the mechanism of KCl is not purely voltage-dependent and is partly due to Ca2+ sensitization. With membrane depolarization there is an increase in intracellular calcium, which activates Ca2+ -calmodulin dependent protein kinase II (CaMKII) which causes phosphorylation of MLC kinase resulting in a reduction in kinase activity for the Ca2+ -calmodulin complex (57). KCl has been found to activate ROK (Rho-associated protein kinase) too. Although the mechanism is unclear, it proves KCl's role in calcium sensitization.

Tadalafil

Tadalafil is a potent, reversible, competitive inhibitor of phosphodiesterase 5 (PDE5), leading to accumulation of cyclic guanosine monophosphate (cGMP). This drug was investigated for the treatment of hypertension and angina pectoris. During the initial human testing it was found to have minimal effect on angina but had an interesting side effect—marked penile erection. GlaxoSmithKline company discovered the product and Eli Lilly company applied for approval of the drug for erectile dysfunction. It was another oral drug approved for this condition after sildenafil. It was called by the trade name "Cialis". Its usage is safe and is shown not to be related to sudden cardiac death or myocardial infarction as earlier believed rather it may be protective in myocardial infarction (14). It is absolutely contraindicated when the patients is already on nitrate therapy because of chance of hypotension. Sildenafil and vardenafil are contraindicated within 24 hours of nitrate therapy. Compared to it tadalafil should not be used within 48 hours of nitrate therapy. Angiotensin receptor blocker (Bosentan) should be used cautiously with tadalafil and dose adjustment of bosentan may be required. It is relatively contraindicated in multitherapy for hypertension and comedication with drugs which increase levels of tadalafil. Potent cytochrome P-450 inhibitor drugs like ketoconazole, itraconazole, erythromycin, HIV protease inhibitors increase plasma tadalafil concentrations (58). It is also been used in the treatment of pulmonary artery hypertension. Tadalafil is more potent for PDE5 than PDE6

(700 times). PDE6 is an enzyme which is normally present in the photosensitive receptors of retina of human beings; Ocular side effect in terms of changes in the perception of color hue and brightness are less in tadalafil compared to sildenafil and vardenafil. The selectivity for the enzyme PDE3 is 10,000 times less than it is for the enzyme PDE5. PDE3 is an important enzyme involved in the regulation of contraction of cardiac muscle. This lesser sensitivity offers protection even in cardiac patients. Headache, flushing, rhinitis, and dyspepsia are other important frequently associated side effects of tadalafil.

Pharmacokinetics and metabolism

Throughout the dosing range from 2.5 mg to 20 mg, tadalafil exposure expressed in terms of AUC, increases proportionally. Tadalafil is absorbed rapidly and it is a highly plasma protein bound drug. This drug is metabolized predominantly by hepatic CYP3A enzyme. This enzyme causes formation of catechol product which after successive methylation and conjugation gives rise to methylcatechol glucuronide, which is a major circulating metabolite. Metabolites are mainly excreted in faeces(61%) and urine (36%). by time of dosing. Exposure was not substantially affected by time of dosing. Food does not have any significant effect on bioavailability.

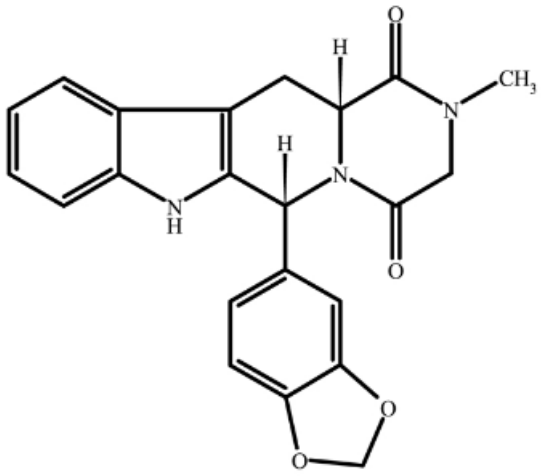


Figure 4. Structure of Tadalafil

Tadalafil has a mean C_{max} (378 µg l^{-1} for 20 mg) observed at 2 h. Following that there is a monoexponential fall in concentration with mean $t_{1/2}$ of 17.5 hours. No clinically significant effects of age, sex, BMI were identified. Following once a day administration, on day 5 steady state concentration was achieved. Duration of action for tadalafil is 36 hours, highest among the other congeners (59).

Pharmacodynamics

Tadalafil has previously been found to be useful for erectile dysfunction to obtain and maintain penile erection for satisfactory sexual intercourse in a variety of causes including diabetes (vasculogenic), spinal cord injury (neuroreflexogenic), and nonorganic (psychogenic) causes. There are other drugs like milrinone, vesnarinone, and enoximone which are specific for cAMP-specific PDE3 inhibitors. These increase long-term

mortality in patients with heart failure. The selectivity of tadalafil for PDE5 is very specific and it does not increase the levels of cAMP and therefore do not cause cardio toxic adverse effects which PDE3 inhibitors are known to cause in the myocardium. To add to this, PDE5, the enzyme which tadalafil primarily inhibits is not present in human myocardium. Tadalafil also acts on PDE11, although the function of this enzyme is yet to be determined. Tadalafil produces a transient modest reduction in systolic (8 to 10 mm Hg) and diastolic (5 to 6 mm Hg) blood pressures. Tadalafil does not inhibit platelet function directly but moderately increase the inhibitory effect of sodium nitroprusside on platelet aggregation due to its requirement for an increase in NO to cause its effects. Patients taking tadalafil have reported recoverable visual problems. The visual problems usually encountered are blue-green vision, blurred vision and a supersensitive perception for light. The adverse effects are mostly dose related and increases with increasing doses.

Iberiotoxin

Venoms being a good source for containing molecules that interacts with ion channels. Peptidyl toxins derived from different sources has the ability to block voltage-dependent ion channels like Na^{+}, K^{+}, $Ca^{2(+)}$ channels. Iberiotoxin belongs to such a toxin that is a specific and potent inhibitor of high conductance, $Ca^{2\,(+)}$ activated potassium (K^{+}) channel.

Iberiotoxin is derived from scorpion Buthus tamulus. It contains 37 amino-acid and has similarity with charybdotoxin. Charybdotoxin is another scorpion derived toxin having similar activity (60). Both of them are inhibitor of high conductance, $Ca^{2(+)}$-activated K+ channel. Iberiotoxin has some allosteric mechanism over charybdotoxin and there is an overall charge difference between these two molecules as iberiotoxin contain four extra acidic amino acid residue and one less basic amino acid group (61). When iberiotoxin is applied to the external side of the membrane it is able to block the skeletal muscle $Ca^{2(+)}$ activated K+ channel that is present in the neutral-planar bilayers. As tetraethylammonium binding causes inhibition of binding of iberiotoxin competitively, this points towards the binding of iberiotoxin to the channel is probably external (62).

Iberiotoxin having a high molecular weight of 4230.85, is soluble in water and to be stored in -20^{o} C

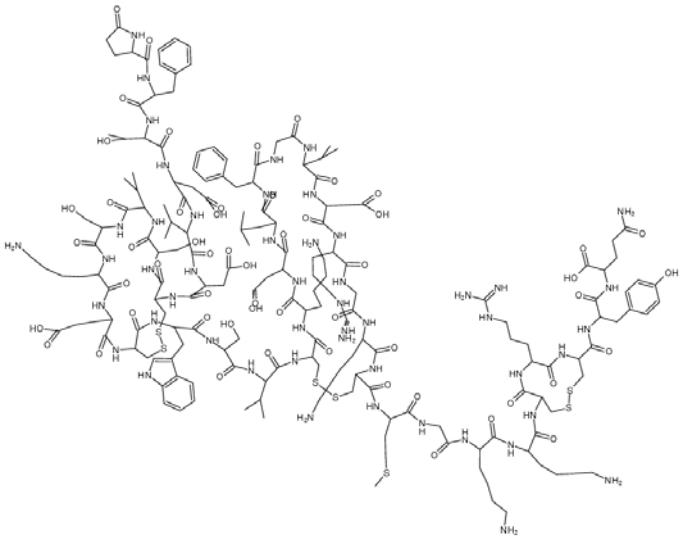


Figure 5. Structure of Iberiotoxin

Iberiotoxin has been used in multiple studies to check the effect of large conductance calcium dependent potassium current (BKCa) in different smooth muscles like rabbit basilar artery smooth muscle (63), bovine aortic smooth muscle (64), human coronary artery (65), urinary bladder (66), myometrium (17), nervous system (67) etc.

Dimethyl sulfoxide (DMSO)

For a long time DMSO is being commercially used as a solvent. DMSO is a byproduct of wood industry. In some animal studies it has been shown that DMSO can cause relaxation of detrusor muscle possibly by altering the $Ca^{2(+)}$ sensitivity of the microfilaments (68). DMSO also may be effective in lung adenocarcinoma by acting on tumor suppressor gene (69).

MATERIALS AND METHODS

MATERIALS AND METHODS

This study was conducted in the Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore. The study was approved by Institutional Review Board and Ethics Committee (Annexure 1).

Inclusion criteria:

- Premenopausal patients
- Patients undergoing operation for benign medical conditions like fibroids, dysfunctional uterine bleeding and prolapse.

Exclusion criteria:

- Patient with malignant conditions
- Post-menopausal women
- Patients refusing informed consent.

Informed Consent:

After fulfilling the inclusion criteria, patients, who were selected for the study, were given informed consent sheet (Annexure).This informed consent form was composed of two part, one being the information sheet for

the patient and the other the consent sheet. Patients own language was used in the informed consent form as well as for conversation with the patient. In the information sheet details about the study were explained. It was clearly mentioned to the patients that the research study will be done in-vitro and it may not give any direct benefit to the patient. It was explained to the patients that even if they are not ready to give consent for the study, their surgery or post-operative hospital stay and treatment will not be hampered anyway. They were given time to discuss with their near relatives if they were in confusion. Their legal right to withdraw at any point of the study at their own will without any consequences was also impressed upon them. All their doubts were clarified to their utmost satisfaction. In case of their willingness to participate in the study one copy of information sheet with investigators signature was given to the patient and one copy of consent form with patient and witness signature was kept for study purpose. Contact information of the investigators were given to the patient in the information sheet for any other further clarification.

Collection and transport of tissue:

Myometrium sample from freshly operated uterus was transported to the Pharmacology laboratory in freshly prepared pre-oxygenated physiological salt solution (PSS).

The methods followed for obtaining, transporting and preparing the tissue were done as suggested by Crankshaw et al (27). Gynecologists were informed to dissect a full thickness uterine mass measuring 2cm × 2cm from the lateral wall of the uterus. The sample thus obtained was immediately transferred to ice-cold physiological salt solution. Then the sample was transported in an icebox filled with icepacks to the pharmacology laboratory within an hour. Once it reached the laboratory the sample was transferred to physiological salt solution (PSS) at room temperature. The PSS used was modified Krebs Henseleit solution.

Tissue dissection and mounting

The obtained sample was examined for orientation of muscle fibers under a magnifying glass. Pathologic changes like fibroids were also looked for. The tissue was made devoid of the serosa and the endometrium. Myometrial strips measuring 3mm × 3mm × 12mm were made from longitudinal incision on the myometrium. Weight of the each myometrial strip was 210 mg +/- 10 mg. One myometrial strip was mounted in a 20 ml organ bath with one end tied to the bottom of the chamber and the other to force transducer. The organ bath was filled with PSS and aerated with oxygen. Oxygen was bubbled through the solution constantly at a rate of 12 bubbles per minute. The temperature of the organ bath was maintained at 37.5°C. A resting tension of 20 milliNewtons (mN) (~2.0g) was applied to the tissue. It was maintained for a period of 45 minutes with frequent

adjustment of length (tissue stretches with tension) with a micromanipulator to keep the resting tension constant at 20 mN. During this 45 minutes the PSS in the bath was continuously washed at intervals of 10 minutes. Tissues which showed spontaneous contraction during this period were considered viable and were subjected to further experiments. An isometric force transducer was used to measure the contractile responses of the myometrium. It was connected to a student's physiograph to record the changes.

Students Physiograph

One end of the tissue was tied to an isometric force transducer (T-305 Force Transducer, Ft-2287, INCO, Ambala, India) and tissue tension sensed by the transducer was recorded using a students physiograph (INCO). A 50 Hz filter was used between the coupler and the amplifier to dampen the artifacts caused by AC power supply and the instrument was earthed. The sensitivity of the physiograph and the balance of the force transduction coupler were not adjusted after initial optimization. After the equilibration period of 45 minutes, the baseline was readjusted and never after during the course of the experiment.

Drugs and Chemicals

Double distilled, water filtered with 22 nm filter (Milli-Q Advantage A10 Water System Production Unit, Millipore) was used to make all

solutions to avoid microbial contamination. KCl (Qualigens, Fisher scientific, Chennai, India) was dissolved in distilled water to have a molarity of 2.5 Mol. KCl was prepared fresh every experiment day. Tadalafil (Santa Cruz biotechnology, Chennai, India) in pure powder form was dissolved in DMSO (Qualigens, Fisher scientific, Chennai, India) to get a working stock molarity of 2 milli mole (mMol). Iberiotoxin (Tocris, Santa Cruz biotechnology, USA) in pure power form was dissolved in distilled water to get a working stock molarity of 4 micromolar (μMol).

Stock solutions of tadalafil and iberiotoxin were stored in a -20^0 centigrade refrigerator. All solutions were not kept for more than a month and fresh solutions made after each month. All the other salts which were used in the making of modified Krebs-Henseleit solution were bought from Qualigens, Fisher scientific, Chennai, India.

Solutions

The physiological salt solution (PSS) used was modified Krebs-Henseleit solution. Its composition was (mMol): NaCl 115.5; KCl 4.6; MgSO4 1.16; NaH2PO4 1.16; CaCl2 2.5; NaHCO3 21.9; and glucose 11.1. The drug concentrations used in the study were KCl 55mM; tadalafil 40 μM; iberiotoxin 100 nano-molar (nMol)

To achieve the desired concentrations of drugs in 20 ml PSS 440 µL of KCl, 400 µL of tadalafil and 500 µL of iberiotoxin was taken from the respective stock solutions.

Selection of dose of tadalafil

A preliminary set of experiments were done to identify the concentration of tadalafil which elicits a minimum appreciable response and the concentration which produces maximum response by logarithmic increments in dose titers.

Selection of dose of iberiotoxin

Iberiotoxin dose was selected from previously done similar studies (17,64,67) as the amount of iberiotoxin was limited. The dose that was used throughout the experiment was 100 nMol.

Experimental Proctedure

The tissue mounted in the organ bath was allowed to equilibrate for a period of 45 minutes with a constant tension of 20 mN. At the end of 45 minutes the tissues which generated less than a force of 2 g contractile force with 55mM KCl were discarded. In case of discarding, a new strip of the same tissue was tried. If the tissue was able to produce enough force, then the entire experiment was carried on. All the drug concentrations mentioned in

this write-up were the concentration attained in the 20ml organ bath when the drug was added to the bath. The stock solutions were made in such a way that not more than 0.5 ml was added to the bath to prevent any dilutional effect. During the entire experiment the organ bath was maintained at 37.5°C by a heater and stirrer. The organ bath was constantly aerated with oxygen at a rate of 12 bubbles per minute. Each contraction was made for a duration of 90 seconds and after each contraction the tissue was washed by changing the PSS in the bath 6 times, each at 3 minute interval, to ensure complete relaxation of the strip. The tissue was given a period of rest of 15 minutes. The KCl contraction was repeated till there was a series of 3 similar looking consecutive contractions. Tissues which did not get similar contractions consecutively with KCl alone were repeated with KCl contractions for 2hrs. If 3 consecutive responses appear similar, then the experiment was continued, otherwise the tissue was discarded. Last KCl response was recorded. This response served as an active control for the next contraction. After a washout period of 15 minutes, the myometrial strip was incubated with 40 μM concentrations of tadalafil in the organ bath for 10 minutes and then subjected to the contractile effect of 55 mmol KCl, with contact time of 90 seconds. Then the myometrial strip was washed six times with PSS to attain the baseline. The contraction curve was recorded on a student's physiograph. Tadalafil was added from the stock solution to the organ bath directly with a micropipette (Biohit Proline, Sartorius Lab systems). A relaxation period of 15 minutes was used between two different concentration curves for the

washout of the drug and for the drug to reach back its normal resting state at 20 mN. The myometrial strip was incubated with tadalafil in the presence specific channel blocker namely iberiotoxin to understand the mode of action of tadalafil. These concentrations were selected because they were shown to cause significant reduction in contractility of the myometrial strip in terms of decreased area under contractile curve and maximum height of contraction.

Chemicals and instruments used in the experiment:

Figure 6: Biohit micropipette used in the experiment

Figure 7: Iberiotoxin used in the experiment

Figure 8: Tadalafil used in the experiment

Measurement of height and area under concentration curve (AUCC)

Height and AUCC was measured in computer using Adobe Acrobat XI software. Statistical tests were used to analyze the tissue response before and after the administration of test drug, and was compared against a set of control specimens.

Statistical Analysis of experimental data

All physiograph tracings were scanned with a scanner at the end of experiment in the constant format. Scanned tracings were analyzed by the software- "Adobe Acrobat XI". Two parameters were measured: area under contractile curve (AUCC) and maximum height of contraction which are indirect measures of contractile force (27). Percentage inhibition values for the reversal of the inhibition were calculated as:

Percentage inhibition =100-

$$\left(\frac{Value\ after\ exposing\ to\ antagonist}{Value\ before\ exposing\ to\ antagonist} \text{ X } 100 \right)$$

In the above equation, value is either taken as the height of the contractile curve or the area under the contractile curve. Statistical analysis of the experimental data was done using nonparametric tests. To compare the

effect of tadalafil on height and AUCC of KCl-induced contractions, the Wilcoxon matched-pair sign rank test was implemented. Percentage inhibition of height and AUCC of KCl-induced contraction by tadalafil in presence or absence of a specific channel blocker iberiotoxin was also compared implementing Wilcoxon matched-pair sign rank test. Statistical significance was considered when the p-value was less than 0.05. All statistical analysis and generation of data plot was done using “R: A Language for Statistical Computing” (version 3.1.1) and R-studio (version 0.98.1062).

Instruments used in the experiment:

Figure 9: Recording of uterine contractions using students physiograph

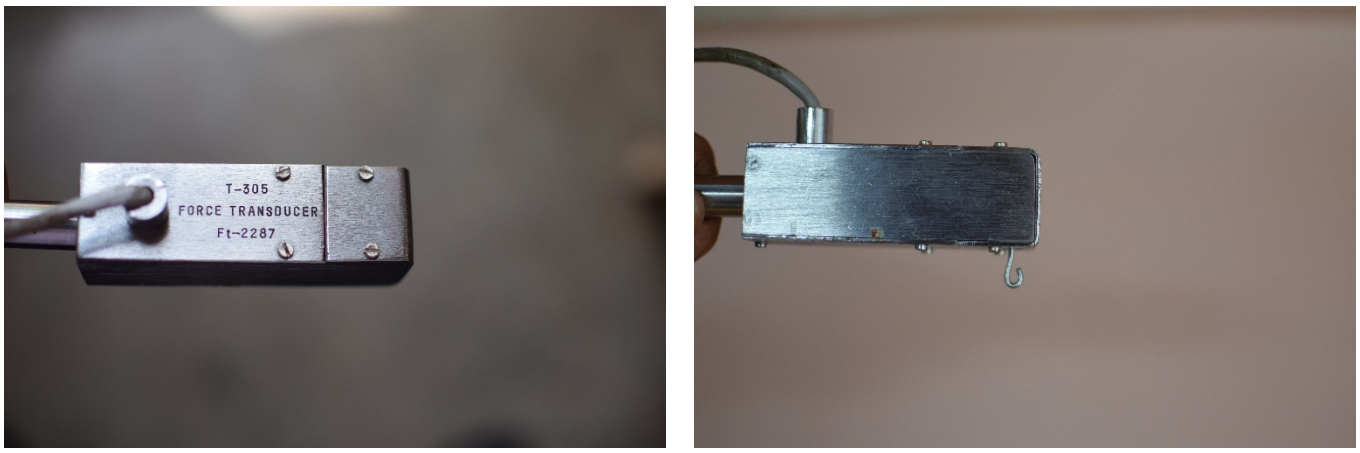
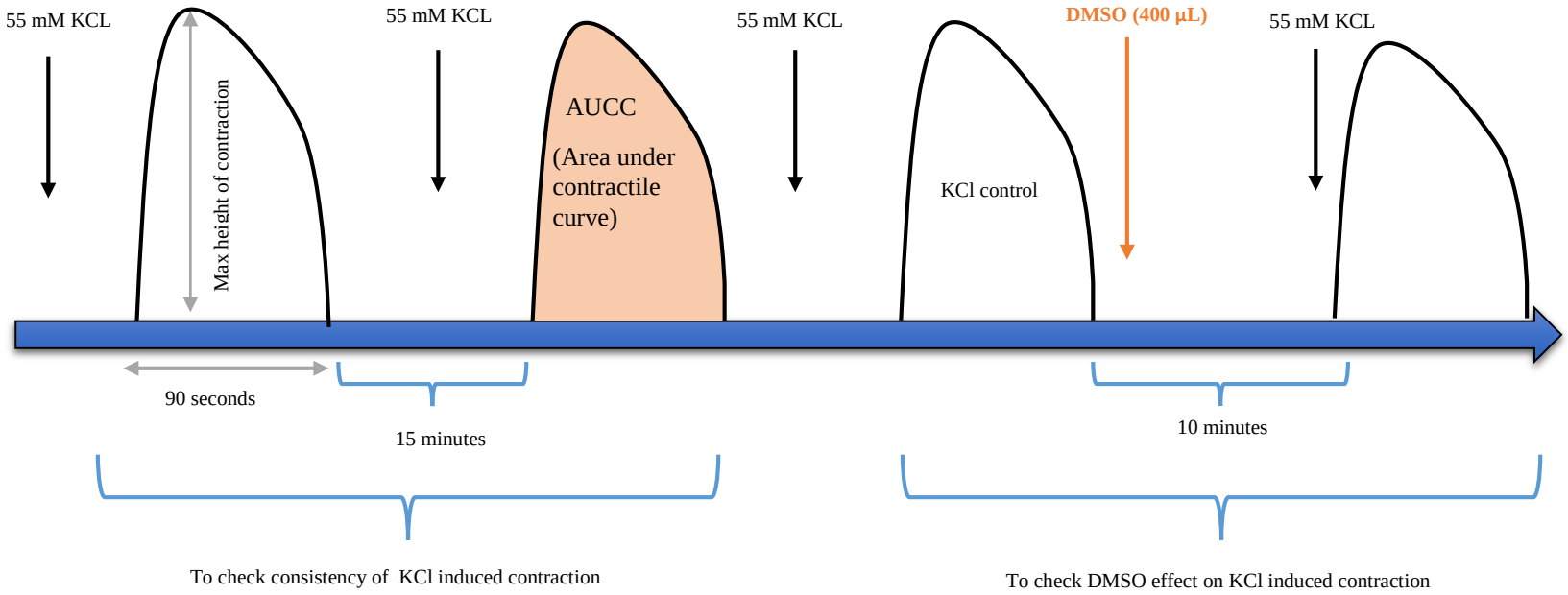
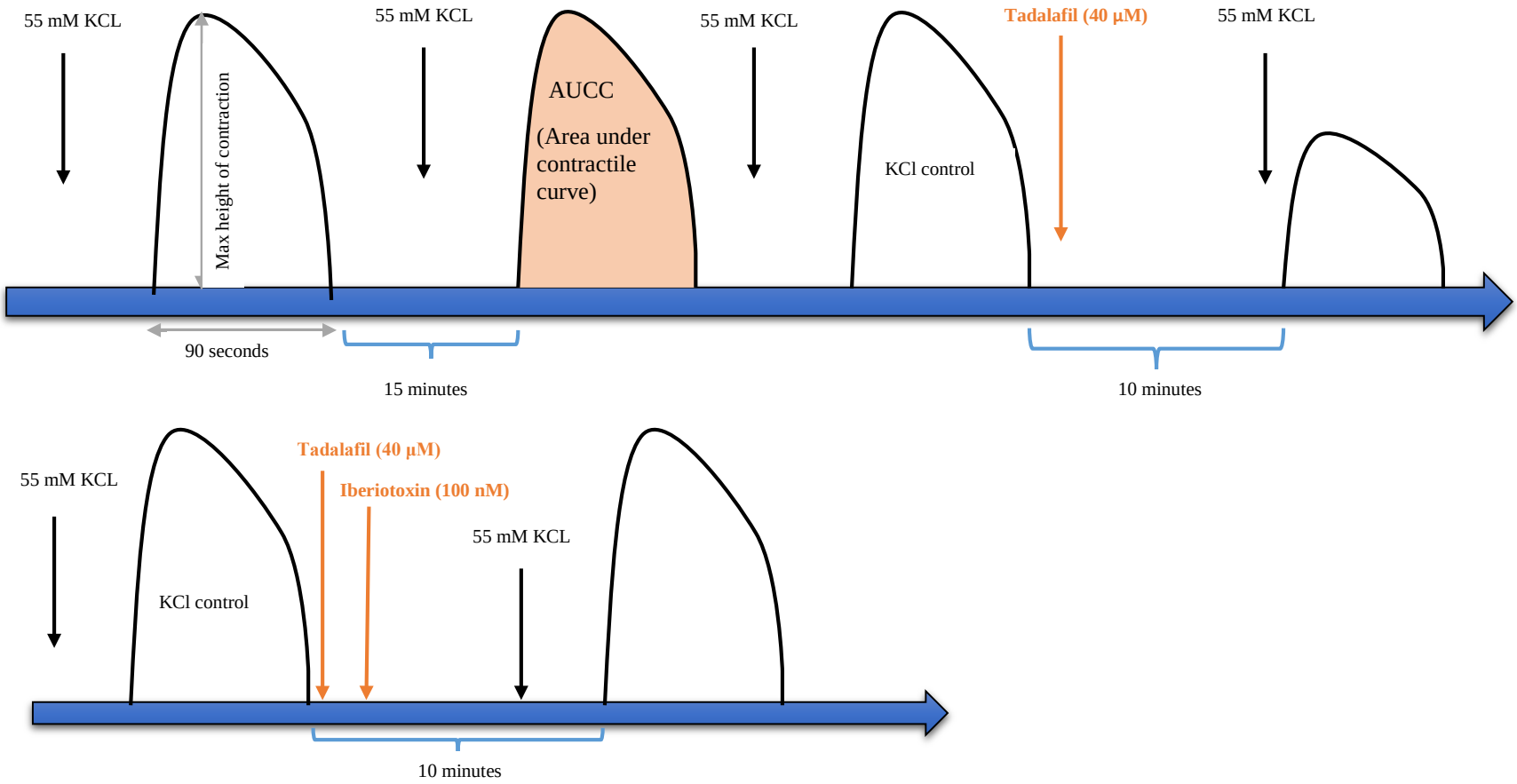


Figure 10: Force transducer

Graphical representation of the methodology (Whether DMSO as a vehicle has any relaxant effect)



Graphical representation of the methodology (Effect of Tadalafil and reversal of tadalafil effect by Iberiotoxin)



RESULTS

RESULTS

The results obtained from the study are summarized here below. The contraction is measured as area under the contractile curve (AUCC) and height of contraction in a 90 second time period.

Demographics

Table 1. Demographics of the study patients

Serial No	Age (Years)	Diagnosis	Surgery performed	Anesthesia
1	48	Adenomyosis	VH	SA
2	44	Uterine leiomyoma	TAH	SA
3	36	Fibroid uterus	TAH with BSO	GA
4	42	Adenomyosis	TAH with BSO	GA
5	40	Fibroid uterus	TAH	SA
6	40	Endometriosis	VH	SA
7	48	Abnormal uterine bleeding - leiomyoma	LAVH with BSO	GA
8	40	Fibroid uterus Endometriosis	TAH with Left Salphingo-oopherectomy	GA
9	45	Fibroid uterus	TAH with BSO	SA
10	42	Submucous fibroid uterus	Total Laparoscopic Hysterectomy	GA
11	42	Abnormal uterine bleeding - leiomyoma	LAVH+BSO	GA

Abbreviations:

TAH: Total abdominal hysterectomy;

BSO: Bilateral Salphingo-oopherectomy

VH: Vaginal hysterectomy;

LAVH: Laparoscopy-assisted vaginal hysterectomy,

SA: Spinal anesthesia;

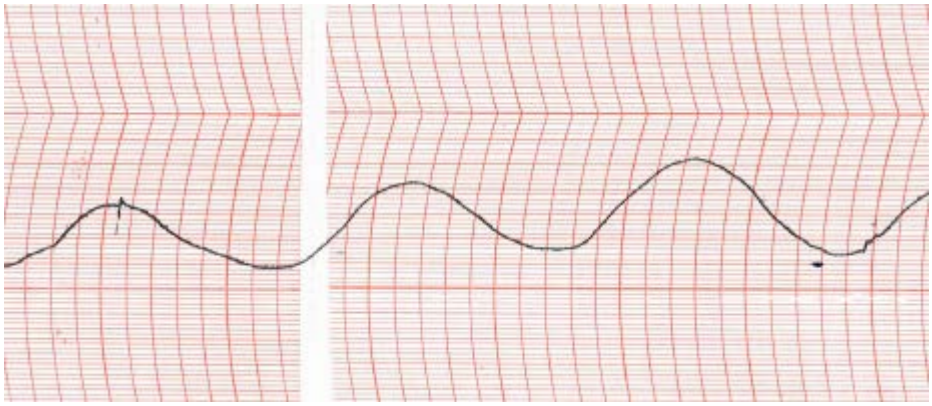
GA: General anesthesia

All the patients were from different part from the country, so they were different in ethnicity. All the patients were operated for benign conditions and they were premenopausal. Samples from the above mentioned patients were viable and there were consistent contractions with KCl.

Viability of tissue

After dissecting the tissue properly with adequate care so that least or no injury happened with the tissue, it is mounted in the organ bath under specified tension. During this stabilization period and even after that spontaneous contraction are noted denoting

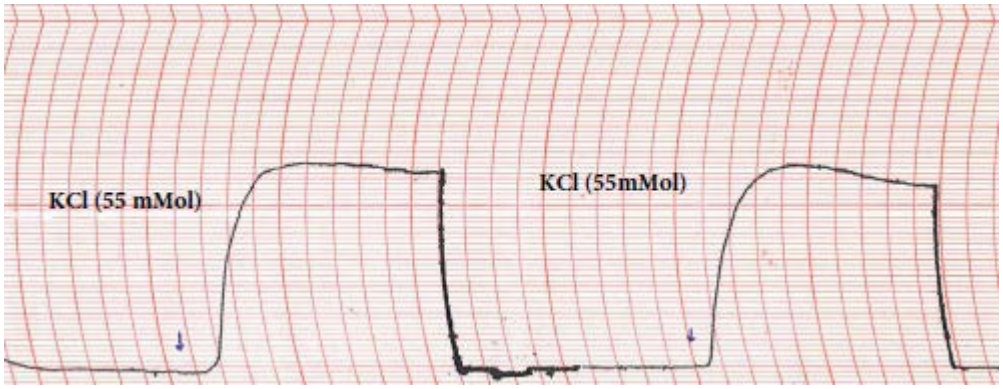
At the end of the 45 minutes resting period, tissues are checked for spontaneous contraction. Tissues which showed spontaneous contraction (Tracing 1) proceeded to the next part of the experiment.



Tracing 1. Spontaneity of myometrial contraction

Consistency of consecutive contractions

Each of the arrow marks indicate the point of KCl addition into the organ bath. The concentration of KCl used was 55mM throughout the experiment. Each contraction was allowed for a period of 90 seconds and then the tissue was washed with PSS allowing the tissue to relax for a period of 15minutes. Then the same procedure is repeated. The consistency in contraction of the three successive contractions are visibly similar both in relating to AUCC and height of the contractions. The samples which could reproduce three consistent successive contractions were used for the rest of the study. The samples which couldn't reproduce this were discarded and therefore not included in the study.



Tracing 2. Consistency of consecutive contractions

Consistency of KCl induced contraction (AUCC)

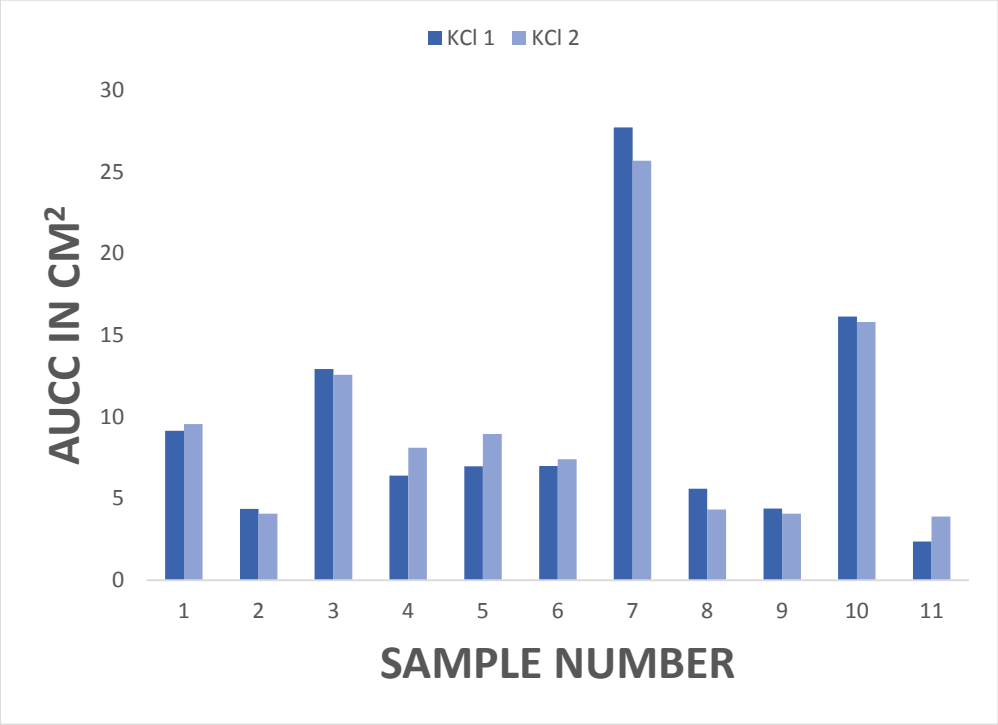


Figure 11. Consistency of AUCC was checked with 2 consecutive contractions. Each set of 2 bars represent a single sample. There was no intra-tissue variability seen in the AUCC of study tissues.

Consistency of KCl induced contraction (Height)

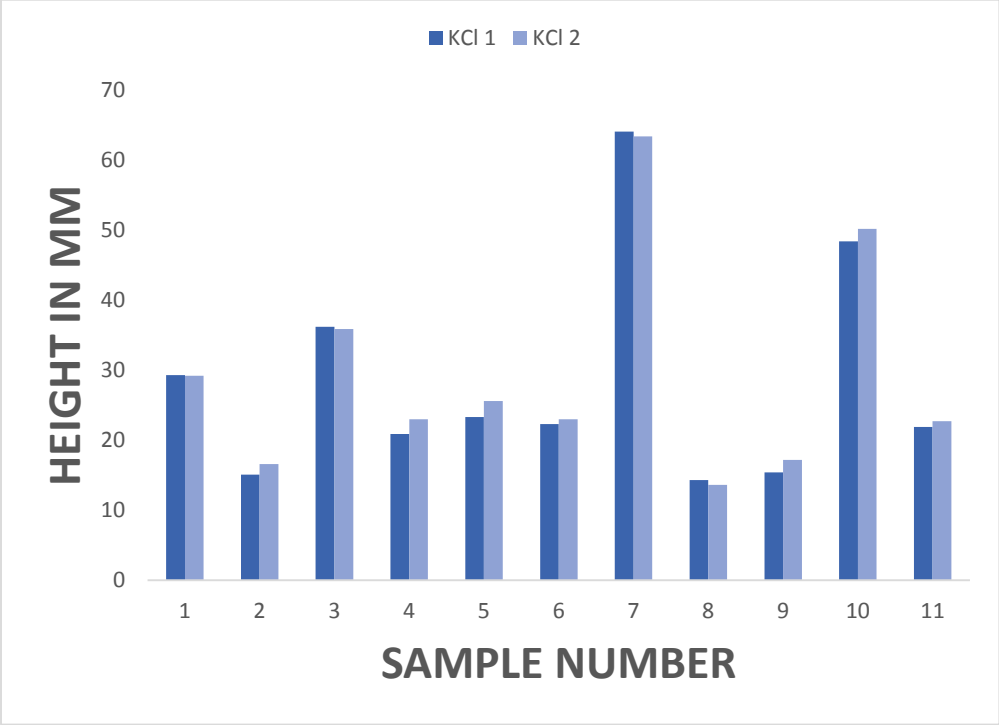


Figure 12 .Consistency of height was checked with 2 consecutive contractions. Each set of 2 bars represent a single sample. There was no intra-tissue variability seen in the height of contraction of study tissues.

Effect of Vehicle (DMSO) on myometrium

Table 2. Effect of DMSO at 400 µL volume on KCl-induced contraction (Height and AUCC)

Drug administration	**Height in mm**	**AUCC in cm^2**
	Mean (SEM)	**Mean (SEM)**
55 mM KCl	33.21 (3.88)	6.86 (0.87)
55 mM KCl + 400 µL DMSO	31.69 (3.63)	6.59 (0.86)
P-value	0.19	0.53

Vehicle Effect

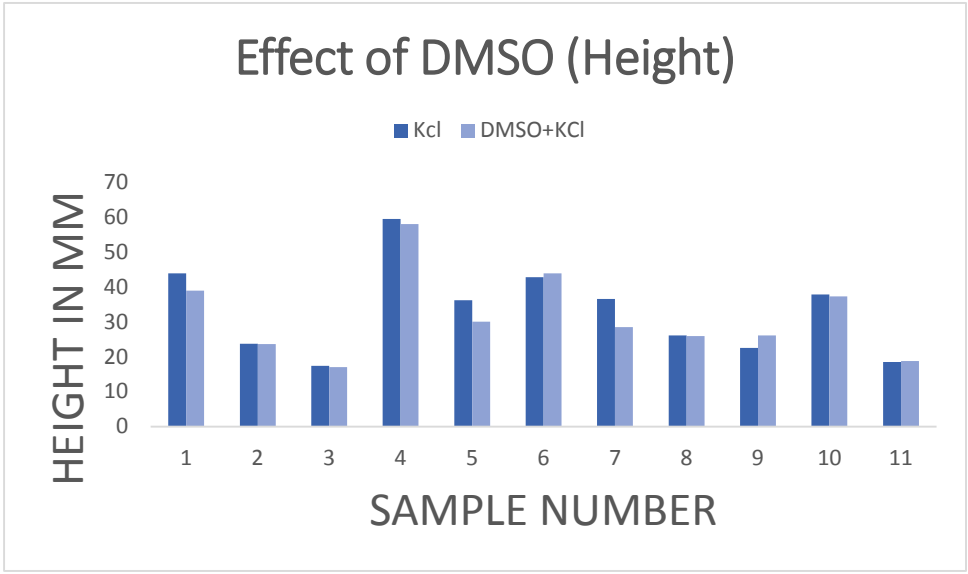


Figure 13. DMSO has minimal effect on height of the contraction induced by KCl. Each pair (2 bars) represents a single sample before and after incubation with DMSO.

Vehicle Effect

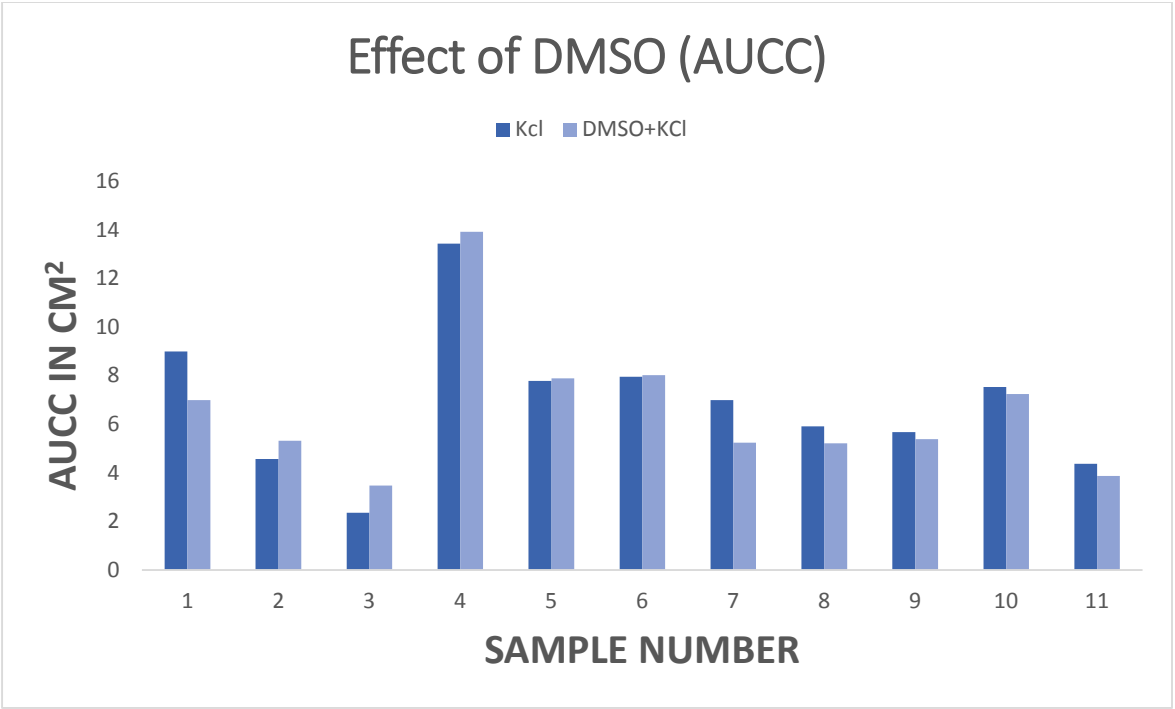


Figure 14. DMSO has minimal effect on AUCC of the contraction induced by KCl. Each pair (2 bars) represents a single sample before and after incubation with DMSO.

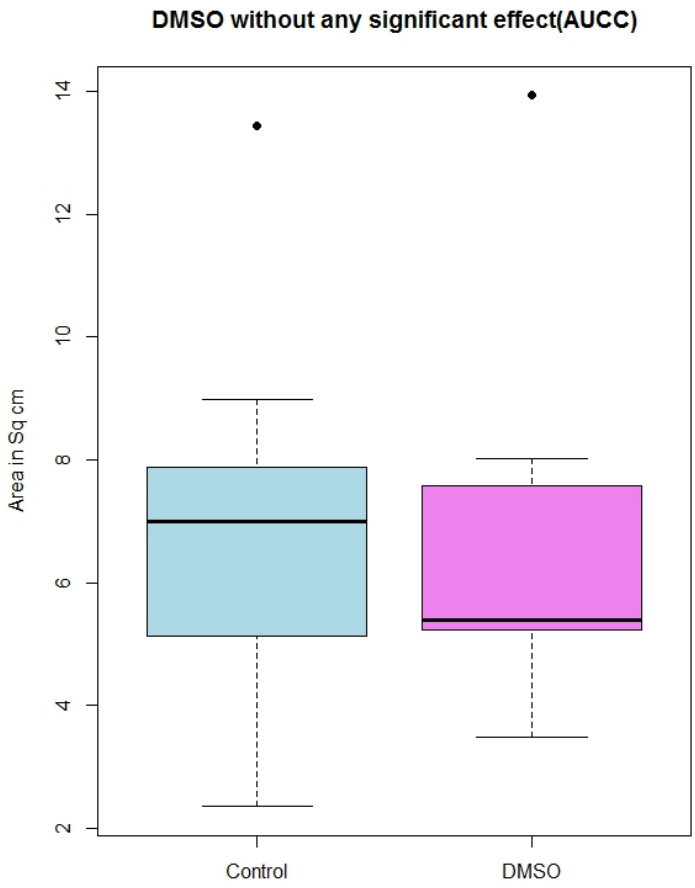


Figure 15. Boxplot showing the median, interquartile range and range of the AUCC of DMSO's effect on the myometrial tissue

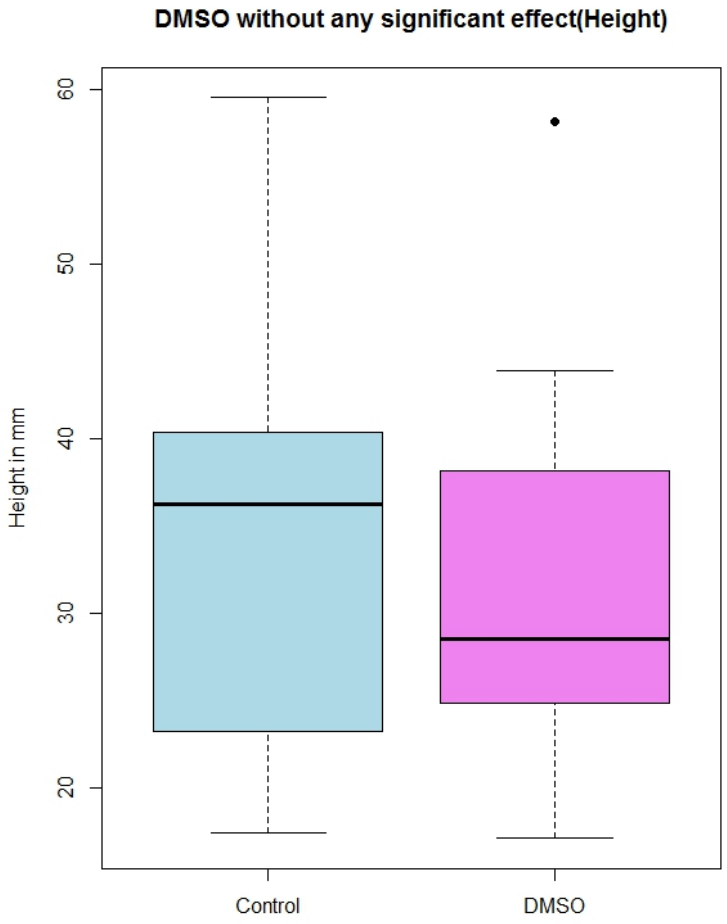


Figure 16. Boxplot showing the median, interquartile range and range of the height of contraction of DMSO's effect on the myometrial tissue

Effect of Tadalafil on myometrium

Table 3. Data of Tadalafil effect at 40 µM concentration on KCl-induced contraction

Tadalafil (40 µM)			
Height (mm)		AUCC (square cm)	
Control	Test	Control	Test
29.72	18.29	10.86	6.44
34.29	29.46	9.08	7.54
36.58	34.54	12.2	10.31
31.75	20.82	10.37	6.73
31.98	39.3	11.29	9.48
28	18.7	10.91	7.33
60.96	52.82	25.6	19.99
15.49	9.14	5.59	3.36
26.12	25.14	11.3	9.55
68.33	65.02	28.89	21.9
32.26	26.92	14.07	9.59

Reversal of Tadalafil-induced relaxation on KCl-induced contraction

Table 4. Data of Iberiotoxin (100 nM) effect on inhibitory effect of 40 µM Tadalafil

Iberiotoxin 100 nM			
Height (mm)		AUCC (square cm)	
Control	Test	Control	Test
32.89	23.07	11.55	7.79
34.49	50.71	8.96	15.59
31.75	30.15	11.81	10.38
21.93	12.56	7.54	4.08
31.98	40	8.61	12.11
20.1	26.72	7.12	7.59
55.62	47.9	21.7	18.07
12.81	11.2	4.56	4.5
18.74	23.7	7.41	8.76
55.52	55.96	20.3	16.83
33.81	33.12	13.83	14.67

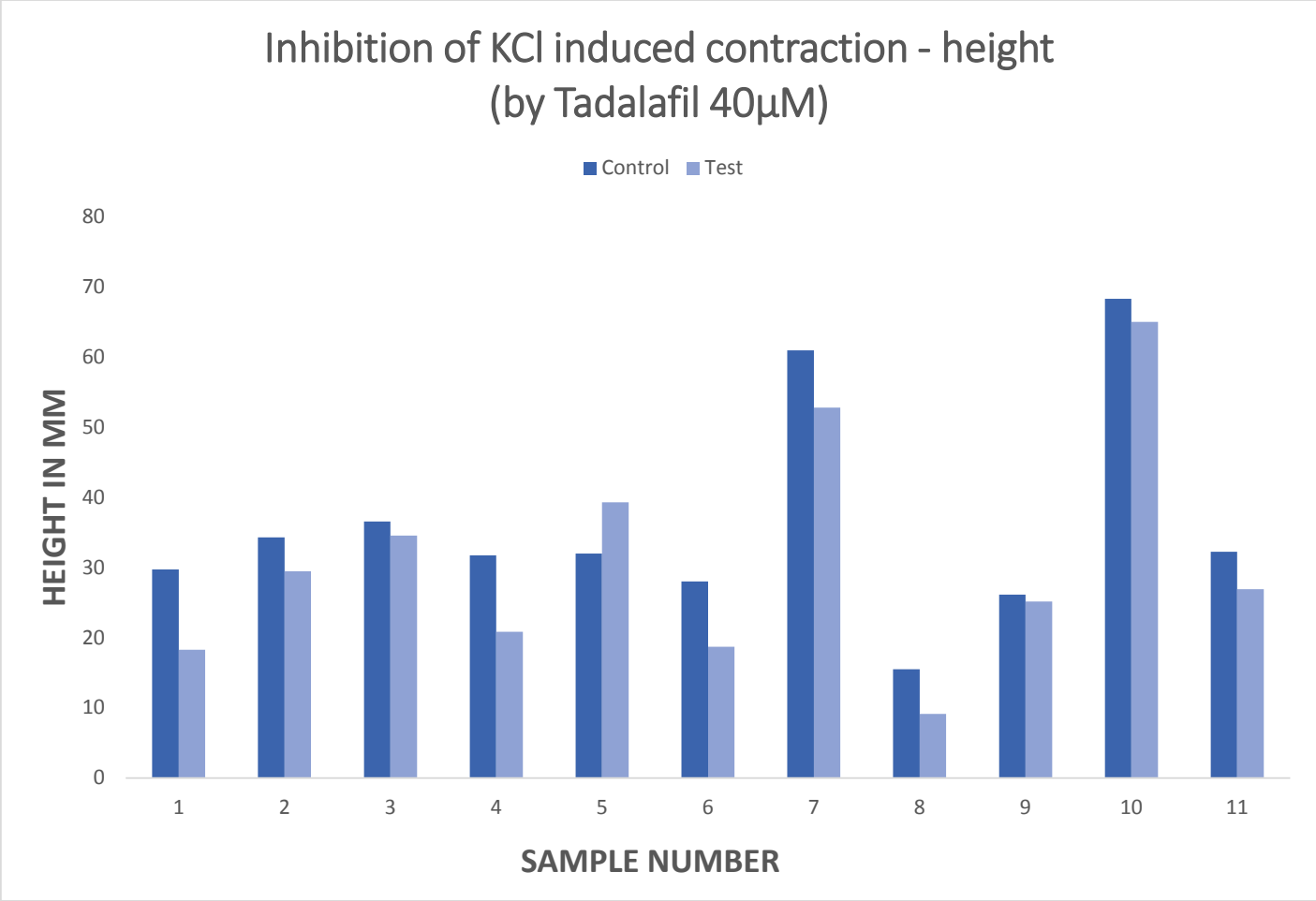


Figure 17. Tadalafil 40 µM inhibits the contraction (Height) induced by KCl. Each set of 2 bars represent a single sample; each before and after adding tadalafil 40 µM.

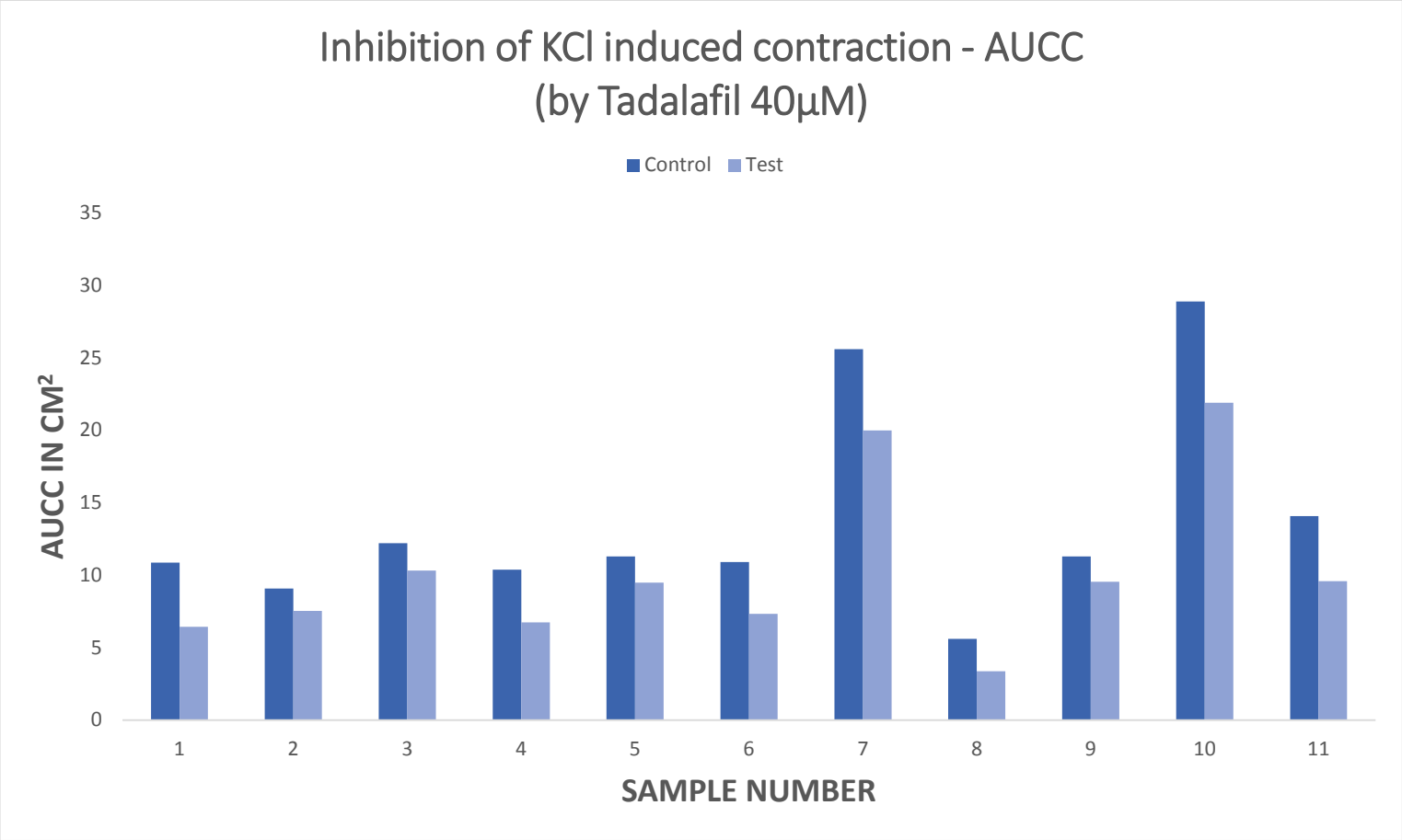


Figure 18. Tadalafil 40 µM inhibits the contraction (AUCC) induced by KCl. Each set of 2 bars represent a single sample; each before and after adding tadalafil 40 µM.

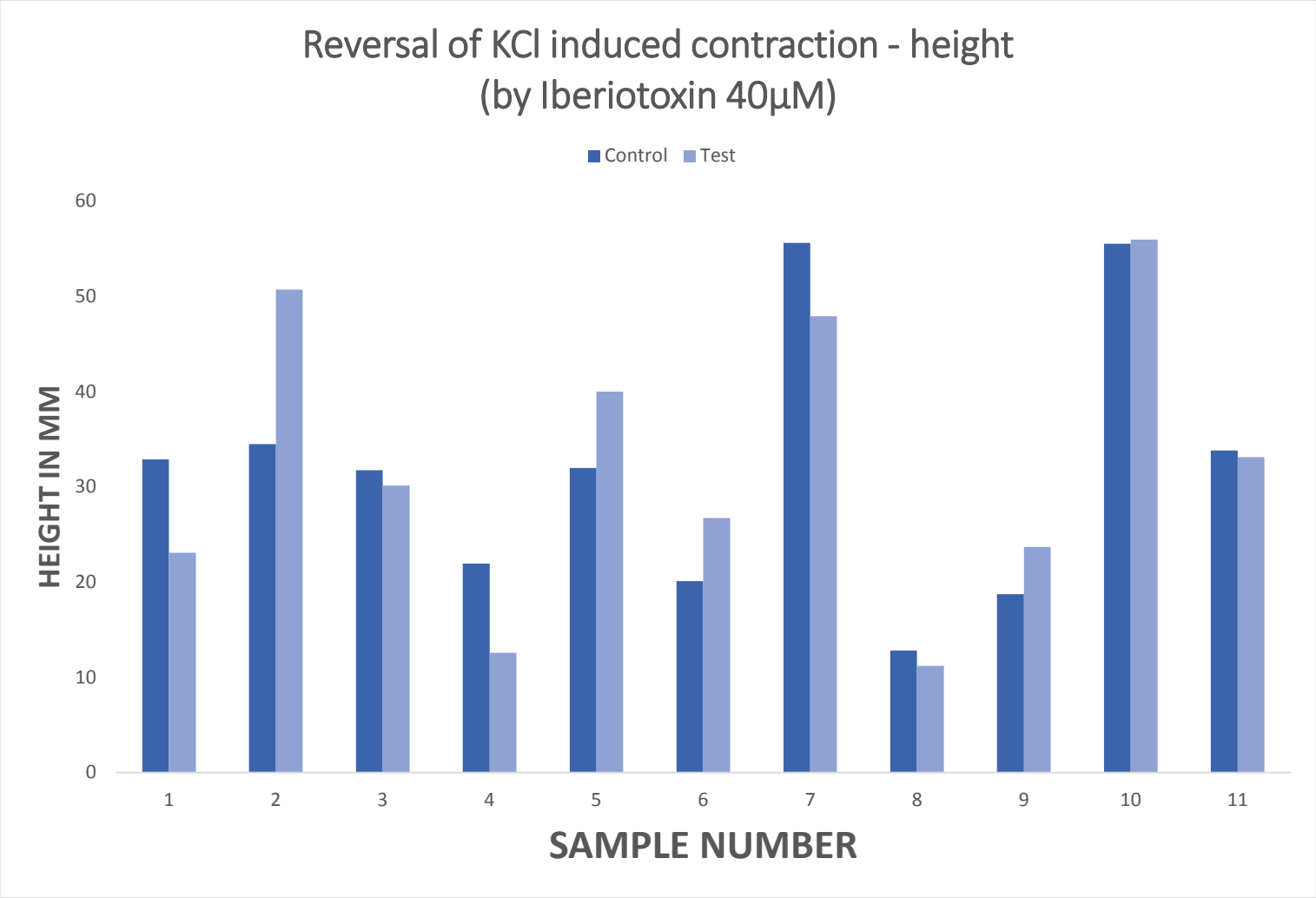


Figure 19. Iberiotoxin 100 nM reverses the contraction (height) induced by KCl. Each set of 2 bars represent a single sample; each before and after adding tadalafil 40 µM and iberiotoxin 100 nM.

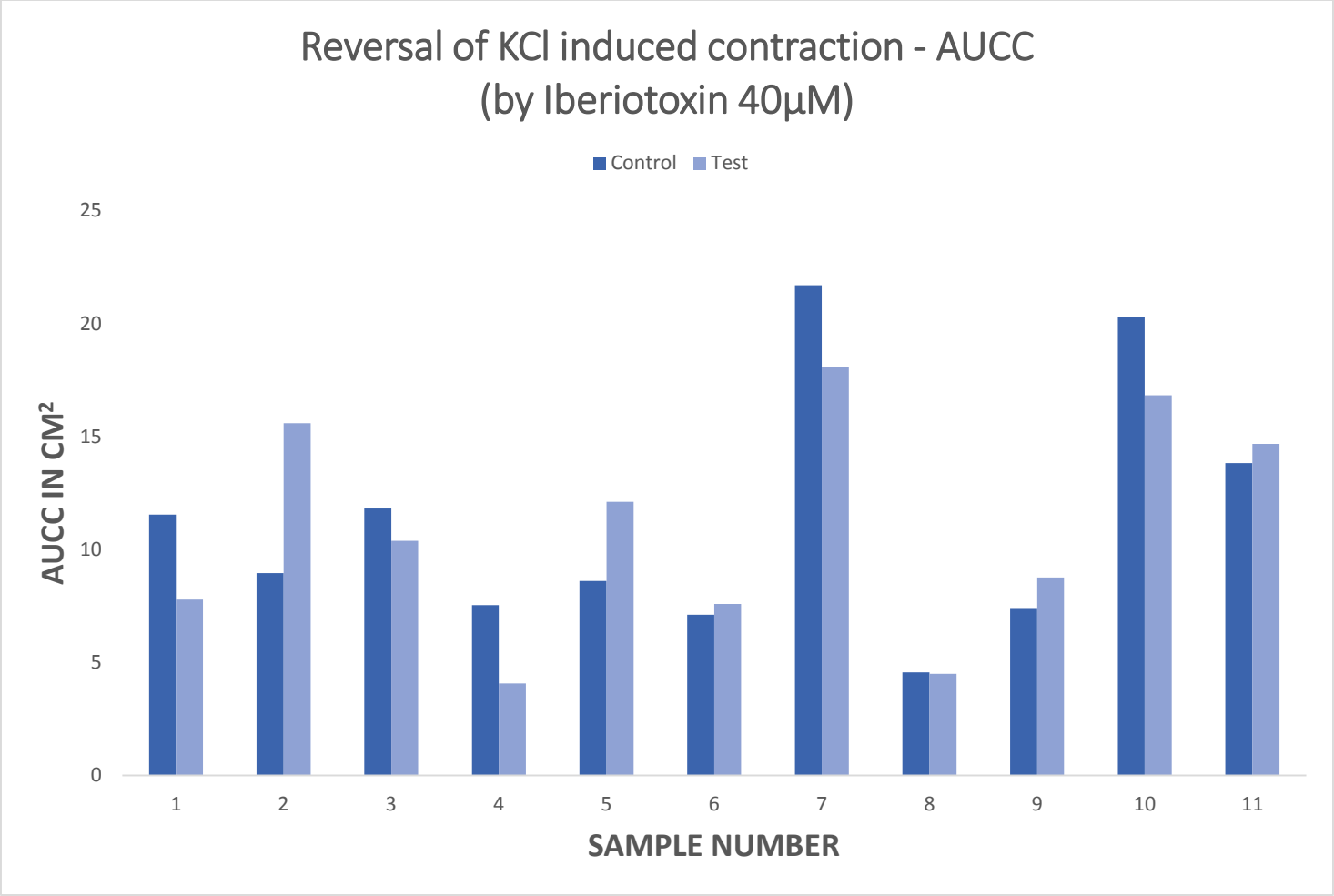


Figure 20. Iberiotoxin 100 nM reverses the contraction (AUCC) induced by KCl. Each set of 2 bars represent a single sample; each before and after adding tadalafil 40 µM and iberiotoxin 100 nM.

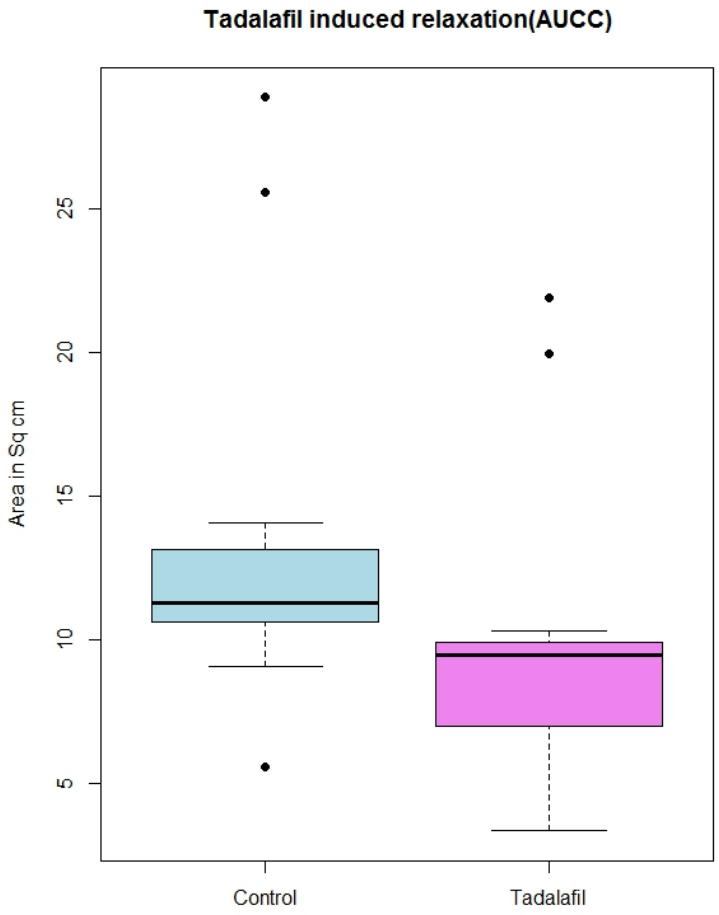


Figure 21. Boxplot showing the median, interquartile range and range of the AUCC of tadalafil's effect on the myometrial tissue

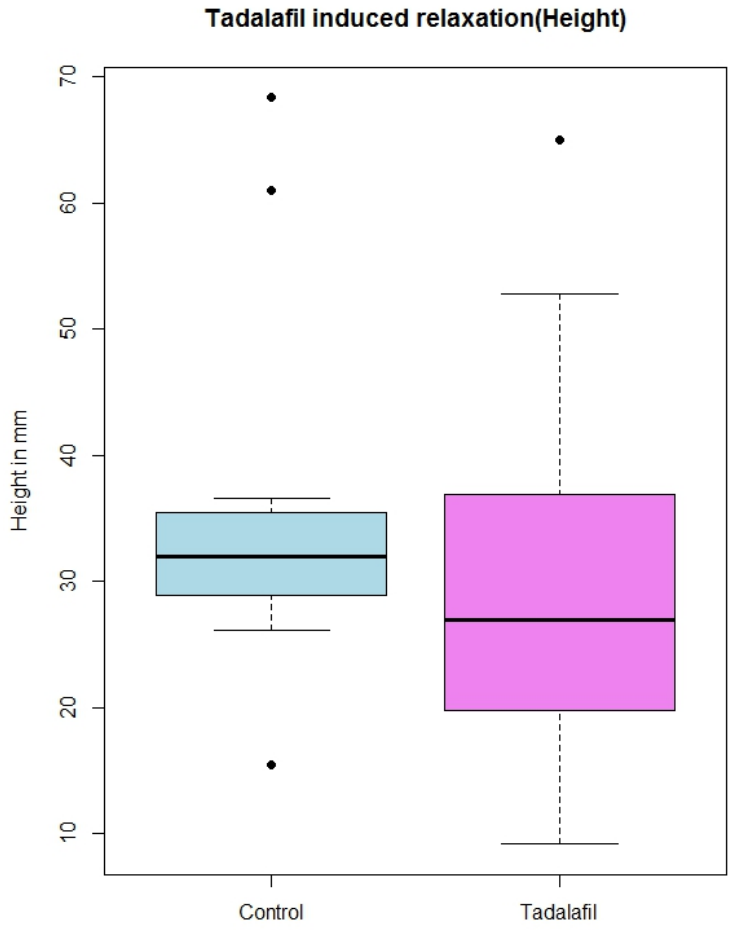


Figure 22. Boxplot showing the median, interquartile range and range of the Height of tadalafil's effect on the myometrial tissue.

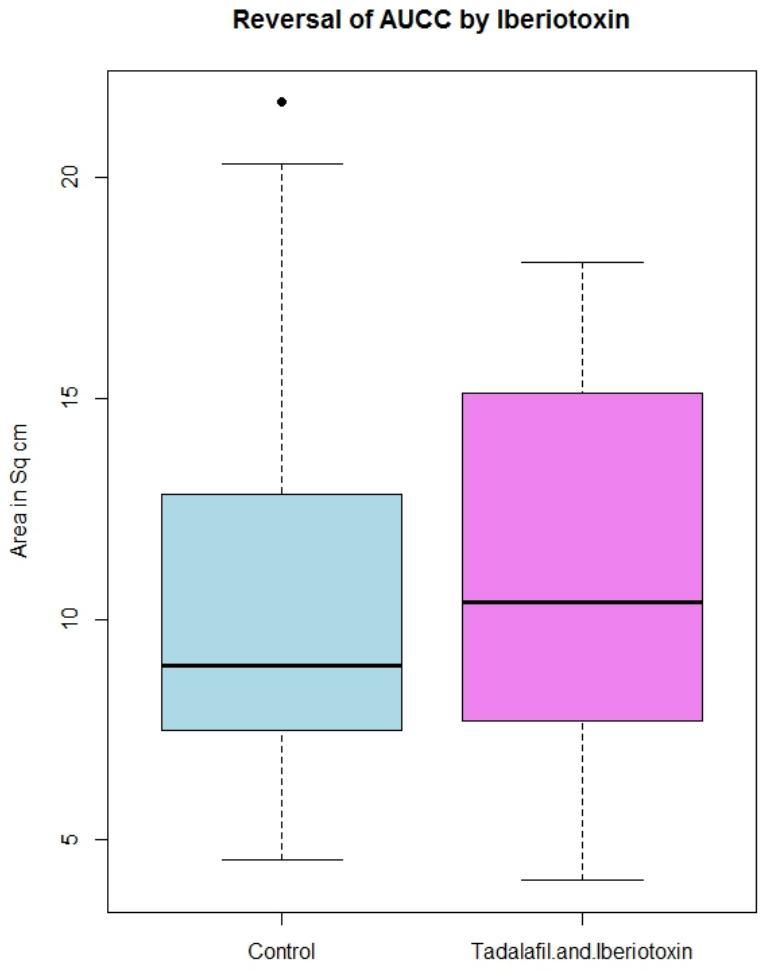


Figure 23. Boxplot showing the median, interquartile range and range of the AUCC of Iberiotoxin and tadalafil's effect on the myometrial tissue

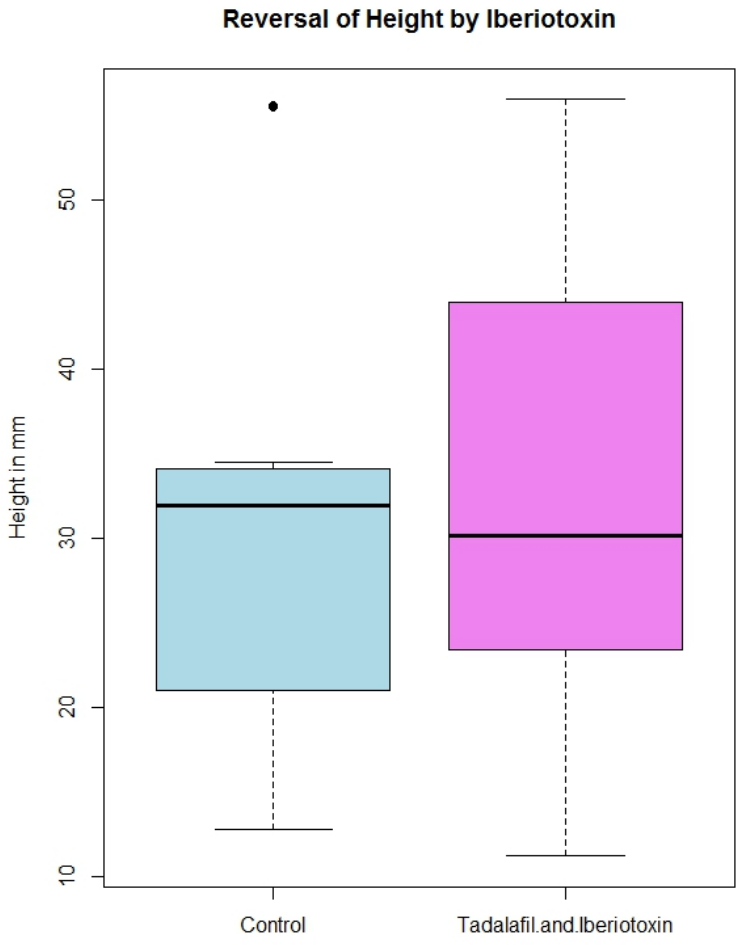


Figure 24. Boxplot showing the median, interquartile range and range of the Height of Iberiotoxin and tadalafil's effect on the myometrial tissue

Inhibitory effect of Tadalafil on non-pregnant myometrium

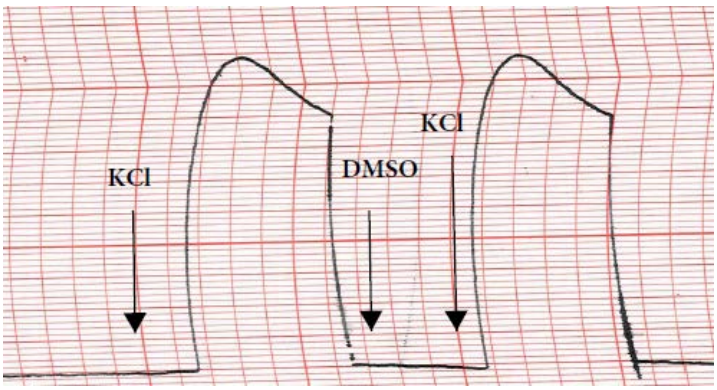
Table 5. Effect of 40 μM tadalafil on KCl-induced contractility of isolated non-pregnant human myometrium tissue (n=11 for each drug administration)

Drug administration	**Height in mm**	**AUCC in cm^2**
	Mean (SEM)	**Mean (SEM)**
55 mM KCl	35.92 (4.61)	13.65 (2.13)
55 mM KCl + 40 μM tadalafil	30.90 (4.92)	10.2 (1.47)
P-value	0.018	0.0009

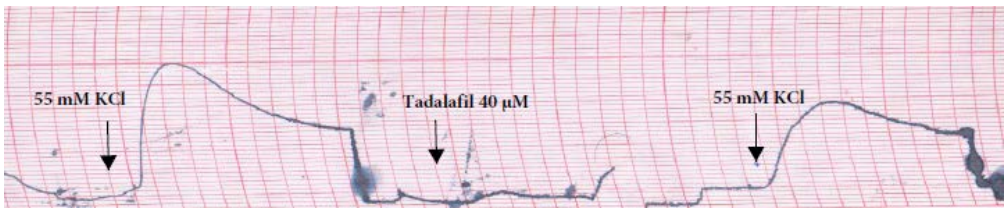
Reversal of tadalafil effect by iberiotoxin

Table 6. Effect of Iberiotoxin on 40 μM tadalafil's inhibition of KCl-induced contractility of isolated non-pregnant human myometrium tissue (n=11 for each drug administration)

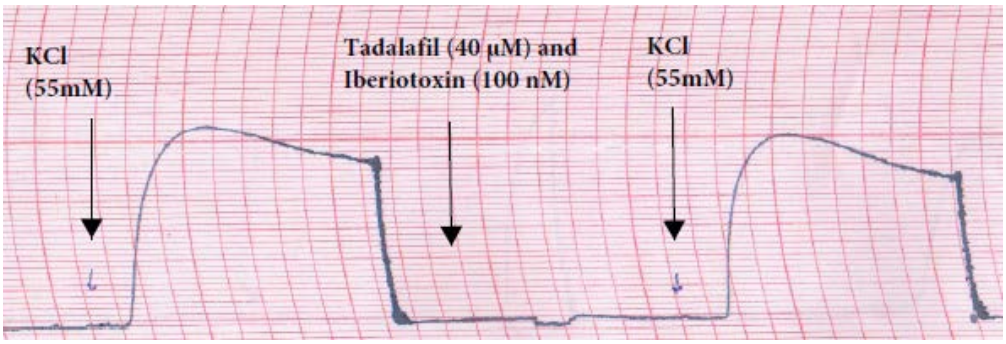
Drug administration	Height in mm	AUCC in cm^2
	Mean (SEM)	Mean (SEM)
55 mM KCl	31.78 (4.16)	11.21 (1.65)
55 mM KCl + 40 μM tadalafil + 100 nM iberiotoxin	32.28 (4.50)	10.94 (1.47)
P-value	0.9	0.7



Tracing 3. Effect of DMSO on 55 mM KCl-induced



Tracing 4. Effect of Tadalafil (40µM) on 55 mM KCl-induced



Tracing 5. Effect of 100 nM Iberiotoxin on 40µM Tadalafil induced relaxation

Table 7. Comparison of median and interquartile range before and after giving tadalafil 40 µM and iberiotoxin 100 nM

Drugs		**Median**	**Interquartile Range**
KCl (55 mM)	Area	11.29	10.62 - 13.14
	Height	31.98	28.86 - 35.44
KCl (55 mM) + Tadalafil (40 µM)	Area	9.48	7.03 - 09.95
	Height	26.92	19.76 - 35.44
KCl (55 mM)	Area	8.96	7.48 - 12.82
	Height	31.98	21.02 - 34.15
KCl (55 mM) + Tadalafil (40 µM) + Iberiotoxin (100 nM)	Area	10.38	7.69 - 15.13
	Height	30.15	23.39 - 43.95

Table 8. Percentage inhibition (median value) by Tadalafil and P-value

Drugs	**% inhibition of tadalafil (Median value)**	**% inhibition of tadalafil and iberiotoxin (Median value)**	**P –value between percentage inhibitions (before and after giving iberiotoxin)**
Area	24.19	1.32	0.009
Height	14.08	2.04	0.01

DISCUSSION

DISCUSSION

Phosphodiesterase 5 inhibitors are known to relax smooth muscles through their action on specific phosphodiesterases increasing the levels of cGMP in the cell (71). This results in an increase in protein kinases which reduce the intracellular calcium levels. Recently the action of other PDE5 inhibitors on the term non-labor pregnant human myometrium was studied by Khan et al (18). Interestingly, the current pattern studied and specific antagonists tested on isolated human myometrium suggested the action of PDE5 inhibitors (tadalafil) on calcium activated potassium channel (BKCa) (18). This suggestion was also strengthened by the fact that the well characterized phosphodiesterase 5 inhibitor zaprinast has no effect on the contractility of the uterine smooth muscle (72). The concentration of tadalafil used in that study (18) was high enough to inhibit the entire family of phosphodiesterases and not just the phosphodiesterase 5. This along with the loss of sensitivity of BKCa receptors to both voltage and intracellular increase in calcium levels in pregnancy (45) prevent the use of tadalafil in pregnancy as tocolytic in preterm labor. However the effect of tadalafil on the human non-pregnant myometrium has not been studied before.

Our results have shown that tadalafil relaxes the non-pregnant human myometrial contraction induced by 55mM KCl. This was observed as a reduction in both the maximal height and area under the contractile curve.

Also, this relaxation with tadalafil was reversed significantly by the addition of 100 nM iberiotoxin.

In similar previous studies it was found that Tetraethylammonium, a non-specific BKCa channel blocker, also increased the spontaneous contractility of myometrial tissue. The relaxant effect of PDE5 inhibitor was not reversed both by the specific adenylate cyclase inhibitor or specific guanylate cyclase inhibitor. In fact an increase in relaxant effect of PDE5 inhibitors on KCl-induced contraction was seen when these agents were added. This suggests that PDE5 inhibitors acts through the calcium-activated potassium channel pathway rather than its phosphodiesterase action. Cyclic adenosine monophosphate (cAMP) is known to be major second messenger involved in the relaxation of human myometrium. It is hydrolyzed mainly by PDE4. Tadalafil and other PDE5 inhibitors are known to have a minimal effect on the cAMP levels in myometrium (73). Also cAMP reduces PDE5 expression in myometrial cells (74). Though the level of effect of tadalafil on cAMP is low, it still can be significant because of the sheer amount of relaxation the cAMP-PKA pathway causes. Cyclic GMP is known to activate BKCa channel as evidenced by the action of cGMP elevating agents on smooth muscle cells previously. This may explain the additional inhibition of contraction as reduced cAMP levels will reduce the baseline activation of BKCa by cAMP. Tadalafil probably acts by a different pathway other than increasing cAMP levels.

Phosphodiesterase 5 inhibitors are inhibitors of the phosphodiesterases involved in the hydrolysis of cyclic guanosine monophosphate (cGMP). This causes an increase in the levels of cGMP, which activates protein kinase G and induces relaxation by decreasing intracellular calcium levels. A study done with various phosphodiesterase inhibitors on human myometrium found that relaxant action of PDE5 and PDE3 inhibitors were minimal and that of PDE4 inhibitors to be significant in combination with β_2-adrenoreceptor agonists (72). Also the amount of PDE4 enzyme present in the cell is significantly more than all the other phosphodiesterase combined. As mentioned before zaprinast, the selective PDE5 inhibitor also doesn't have any effect on myometrial contractility (72). The amount of cGMP expressed in myometrium is least in the non-pregnant state when compared with a term uterus or more so with a preterm uterus (71) although the sensitivity of myometrium to cGMP decreases throughout pregnancy (not only at term) (75). The importance of cGMP in uterine quiescence during pregnancy is yet unclear. The data available on cGMP in non-pregnant myometrium is little and whatever present is also in relative to pregnant myometrium. In one previous study it has been shown by Winston, et al that, oxadiazolo quinoxalin-1- (ODQ) a selective irreversible inhibitor of soluble guanylyl cyclase, decrease its activity. In that study it came was concluded that the absence of reversal of inhibition in the presence of ODQ showed the independence of sildenafil-induced inhibition on the cGMP pathway (17). Cyclic GMP stimulates PKG which may activate the BKCa ,

lack of which may have resulted in the increased inhibition seen when given along with tadalafil.

Tetraethylammonium, the quaternary ammonium cation, acts on the BKCa receptor only at lower concentrations (1-2mM) (76). TEA 1mM has been shown to block BKCa and the same effect could be reversed with verapamil, showing that TEA activates voltage gated calcium channels through its action on BKCa. It inhibits other types of potassium channels at higher concentrations. Studies done previously with 20mM tetraethylammonium have also shown to reverse PDE5 inhibitor-induced relaxation of the contracted myometrium (18). But that effect cannot be attributed to BKCa channel because of the non-specific nature of the dose. Voltage-gated potassium channels get reversed by 10mM TEA (77). This shows that the proposed action through BKCa receptors, although the major mechanism, is probably not the only mechanism by which tadalafil acts. Spontaneous contractions induced by BKCa blockers is a known phenomenon and was also seen in the present study (72)

CONCLUSION

CONCLUSION

In conclusion, we can say, this study has shown that suitably low concentrations of tadalafil inhibit the contractility of the isolated non‑pregnant human myometrium. The likely mechanism that is involved is by opening of the BKCa channel, as suggested from the results. The results also suggest that tadalafil has the potential to be used in clinical conditions like preterm labor or dysmenorrhea, requiring inhibition of myometrial contractility.

LIMITATION OF THE STUDY

The semiquantitative nature of the study can be a limitation of this study.

Isolated myometrium contractility may vary with age, disease condition or hormonal therapy. KCl was the only agonist used, which can give a different result from a pharmacological agonist like oxytocin a GPCR agonists which will contribute to Ca2+ -sensitization also.

The electromechanical coupling of KCl does not involve G-proteins which happens in pharmacomechanical coupling (78). However recent studies have shown KCl's involvement in Ca2+ -sensitization (57).

Further endometrium has a number of celltypes which secrete multiple substances which affect the contraction of myometrium (79). Removing endometrium is necessary to sort out the various actions of the test substance on the myometrium, at the same time also fails to give the net effect as it would happen normally in vivo.

FUTURE SCOPE

Future Scope

Tadalafil has good uterine relaxing properties. It has been shown to be safe in pregnant women too. Clinical trials are required to prove its practical use as tocolytic in preterm labor and also as a spasmolytic in dysmenorrhea. More BKCa channel blockers should also be employed in such studies in future.

Bibliography

1. Dencker A, Berg M, Bergqvist L, Ladfors L, Thorsén LS, Lilja H. Early versus delayed oxytocin augmentation in nulliparous women with prolonged labour--a randomised controlled trial. BJOG Int J Obstet Gynaecol. 2009 Mar;116(4):530–6.

2. Proctor M, Farquhar C. Diagnosis and management of dysmenorrhoea. BMJ. 2006 May 13;332(7550):1134–8.

3. French L. Dysmenorrhea. Am Fam Physician. 2005 Jan 15;71(2):285–91.

4. Moya RA, Moisa CF, Morales F, Wynter H, Ali A, Narancio E. Transdermal glyceryl trinitrate in the management of primary dysmenorrhea. Int J Gynaecol Obstet Off Organ Int Fed Gynaecol Obstet. 2000 May;69(2):113–8.

5. Andersson KE, Ulmsten U. Effects of nifedipine on myometrial activity and lower abdominal pain in women with primary dysmenorrhoea. Br J Obstet Gynaecol. 1978 Feb;85(2):142–8.

6. Benassi L, Barletta FP, Baroncini L, Bertani D, Filippini F, Beski L, et al. Effectiveness of magnesium pidolate in the prophylactic treatment of primary dysmenorrhea. Clin Exp Obstet Gynecol. 1992;19(3):176–9.

7. Proctor ML, Murphy PA. Herbal and dietary therapies for primary and secondary dysmenorrhoea. Cochrane Database Syst Rev. 2001;(3):CD002124.

8. Slattery MM, Morrison JJ. Preterm delivery. Lancet Lond Engl. 2002 Nov 9;360(9344):1489–97.

9. MB et al B. Preterm Labor: A Review. South Asian Federation of Obstetrics and Gynecology,. (September-December 2009;1(3):1-4).

10. de Heus R, Mulder EJH, Visser GHA. Management of preterm labor: atosiban or nifedipine? Int J Womens Health. 2010 Aug 9;2:137–42.

11. Crowther CA, Hiller JE, Doyle LW. Magnesium sulphate for preventing preterm birth in threatened preterm labour. Cochrane Database Syst Rev. 2002;(4):CD001060.

12. Tan TC, Devendra K, Tan LK, Tan HK. Tocolytic treatment for the management of preterm labour: a systematic review. Singapore Med J. 2006 May;47(5):361–6.

13. Croxtall JD, Lyseng-Williamson KA. Tadalafil: in pulmonary arterial hypertension. Drugs. 2010 Mar 5;70(4):479–88.

14. Sesti C, Florio V, Johnson EG, Kloner RA. The phosphodiesterase-5 inhibitor tadalafil reduces myocardial infarct size. Int J Impot Res. 2006 Jul 20;19(1):55–61.

15. Kloner RA, Hutter AM, Emmick JT, Mitchell MI, Denne J, Jackson G. Time course of the interaction between tadalafil and nitrates. J Am Coll Cardiol. 2003 Nov 19;42(10):1855–60.

16. Zhang LT, Park JK. Are phosphodiesterase type 5 inhibitors effective for the management of lower urinary symptoms suggestive of benign prostatic hyperplasia? World J Nephrol. 2015 Feb 6;4(1):138–47.

17. Winston AB, Vazhudhi K, Sen S, Thomas E, Benjamin S, Peedicayil J. Inhibition by sildenafil of contractility of isolated non-pregnant human myometrium. J Pharmacol Pharmacother. 2015 Sep;6(3):136–41.

18. Khan RN, Hamoud H, Warren A, Wong LF, Arulkumaran S. Relaxant action of sildenafil citrate (Viagra) on human myometrium of pregnancy. Am J Obstet Gynecol. 2004 Jul;191(1):315–21.

19. Medina P, Segarra G, Torondel B, Chuan P, Domenech C, Vila JM, et al. Inhibition of neuroeffector transmission in human vas deferens by sildenafil. Br J Pharmacol. 2000 Nov;131(5):871–4.

20. Khan RN, Smith SK, Morrison JJ, Ashford ML. Ca2+ dependence and pharmacology of large-conductance K+ channels in nonlabor and labor human uterine myocytes. Am J Physiol. 1997 Nov;273(5 Pt 1):C1721–31.

21. RN Miftahof, Hong Gil Nam. Biomechanics of the Gravid Human Uterus. Springer. (2011).

22. Standring S. Gray's Anatomy: The Anatomical Basis of Clinical Practice. Churchill Livingstone/Elsevier; 2008. 1551 p.

23. Young RC, Hession RO. Three-dimensional structure of the smooth muscle in the term-pregnant human uterus. Obstet Gynecol. 1999 Jan;93(1):94–9.

24. Senchyna M, Crankshaw DJ. Operational correlates of prostanoid TP receptor expression in human non-pregnant myometrium are unaffected by excision site or menstrual cycle status of the donor. Br J Pharmacol. 1999 Dec;128(7):1524–8.

25. Wikland M, Lindblom B, Wiqvist N. Myometrial response to prostaglandins during labor. Gynecol Obstet Invest. 1984;17(3):131–8.

26. Ascher-Landsberg J, Saunders T, Phillippe M. The role of diacylglycerol as a modulator of oxytocin-stimulated phasic contractions in myometrium from pregnant and nonpregnant rats. Am J Obstet Gynecol. 2000 Apr;182(4):943–9.

27. Crankshaw DJ. Pharmacological techniques for the in vitro study of the uterus. J Pharmacol Toxicol Methods. 2001 Apr;45(2):123–40.

28. Gabella G. Structural apparatus for force transmission in smooth muscles. Physiol Rev. 1984 Apr;64(2):455–77.

29. Word RA, Stull JT, Casey ML, Kamm KE. Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. J Clin Invest. 1993 Jul;92(1):29–37.

30. Savineau JP, Marthan R. Modulation of the calcium sensitivity of the smooth muscle contractile apparatus: molecular mechanisms, pharmacological and pathophysiological implications. Fundam Clin Pharmacol. 1997;11(4):289–99.

31. Marshall JM. Regulation of activity in uterine smooth muscle. Physiol Rev Suppl. 1962 Jul;5:213–27.

32. Sergeant GP, Hollywood MA, McCloskey KD, Thornbury KD, McHale NG. Specialised pacemaking cells in the rabbit urethra. J Physiol. 2000 Jul 15;526 Pt 2:359–66.

33. Young RC, Mathur SP. Focal sarcoplasmic reticulum calcium stores and diffuse inositol 1,4,5-trisphosphate and ryanodine receptors in human myometrium. Cell Calcium. 1999 Aug;26(1-2):69–75.

34. Asbóth G, Phaneuf S, López Bernal AL. Prostaglandin E receptors in myometrial cells. Acta Physiol Hung. 1997 1998;85(1):39–50.

35. Zaccolo M. cAMP and cGMP Signaling Cross-Talk Role of Phosphodiesterases and Implications for Cardiac Pathophysiology. Circ Res. 100(2007):;100:156.

36. Keef KD, Hume JR, Zhong J. Regulation of cardiac and smooth muscle Ca(2+) channels (Ca(V)1.2a,b) by protein kinases. Am J Physiol Cell Physiol. 2001 Dec;281(6):C1743–56.

37. Collins PL, Moore JJ, Lundgren DW, Choobineh E, Chang SM, Chang AS. Gestational changes in uterine L-type calcium channel function and expression in guinea pig. Biol Reprod. 2000 Nov;63(5):1262–70.

38. Forman A, Andersson KE, Maigaard S. Effects of calcium channel blockers on the female genital tract. Acta Pharmacol Toxicol (Copenh). 1986;58 Suppl 2:183–92.

39. Arnaudeau S, Leprêtre N, Mironneau J. Oxytocin mobilizes calcium from a unique heparin-sensitive and thapsigargin-sensitive store in single myometrial cells from pregnant rats. Pflüg Arch Eur J Physiol. 1994 Aug;428(1):51–9.

40. Sanborn BM. Relationship of ion channel activity to control of myometrial calcium. J Soc Gynecol Investig. 2000 Feb;7(1):4–11.

41. Mandi G, Sarkar SN, Mishra SK, Raviprakash V. Effects of calcium channel blocker, mibefradil, and potassium channel opener, pinacidil, on the contractile response of mid-pregnant goat myometrium. Indian J Exp Biol. 2005 Sep;43(9):795–801.

42. Putney JW. Physiological mechanisms of TRPC activation. Pflüg Arch Eur J Physiol. 2005 Oct;451(1):29–34.

43. Khan RN, Matharoo-Ball B, Arulkumaran S, Ashford MLJ. Potassium Channels in the Human Myometrium. Exp Physiol. 2001 Mar 1;86(2):255–64.

44. Heginbotham L, Lu Z, Abramson T, MacKinnon R. Mutations in the K+ channel signature sequence. Biophys J. 1994 Apr;66(4):1061–7.

45. Khan RN, Smith SK, Morrison JJ, Ashford ML. Properties of large-conductance K+ channels in human myometrium during pregnancy and labour. Proc Biol Sci. 1993 Jan 22;251(1330):9–15.

46. Ransom CB, Sontheimer H. BK channels in human glioma cells. J Neurophysiol. 2001 Feb;85(2):790–803.

47. Tseng-Crank J, Godinot N, Johansen TE, Ahring PK, Strøbaek D, Mertz R, et al. Cloning, expression, and distribution of a Ca(2+)-activated K+ channel beta-subunit from human brain. Proc Natl Acad Sci U S A. 1996 Aug 20;93(17):9200–5.

48. Knock GA, Smirnov SV, Aaronson PI. Voltage-gated K+ currents in freshly isolated myocytes of the pregnant human myometrium. J Physiol. 1999 Aug 1;518 (Pt 3):769–81.

49. Smith RC, McClure MC, Smith MA, Abel PW, Bradley ME. The role of voltage-gated potassium channels in the regulation of mouse uterine contractility. Reprod Biol Endocrinol RBE. 2007;5:41.

50. Kidd MW, Leo MD, Bannister JP, Jaggar JH. Intravascular pressure enhances the abundance of functional Kv1.5 channels at the surface of arterial smooth muscle cells. Sci Signal. 2015;8(390):ra83.

51. Hamada Y, Nakaya Y, Hamada S, Kamada M, Aono T. Activation of K+ channels by ritodrine hydrochloride in uterine smooth muscle cells from pregnant women. Eur J Pharmacol. 1994 Dec 15;288(1):45–51.

52. Martin C, Arnaudeau S, Jmari K, Rakotoarisoa L, Sayet I, Dacquet C, et al. Identification and properties of voltage-sensitive sodium channels in smooth muscle cells from pregnant rat myometrium. Mol Pharmacol. 1990 Nov;38(5):667–73.

53. Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. Am J Physiol Cell Physiol. 2005 Apr;288(4):C769–83.

54. Crankshaw DJ, Gaspar V. Pharmacological characterization in vitro of prostanoid receptors in the myometrium of nonpregnant ewes. J Reprod Fertil. 1995 Jan;103(1):55–61.

55. Parimala K, Peedicayil J, Peedicayil A, Nelson N, Ernest K. The effect of a nicorandil congener on isolated human myometrium. Eur J Obstet Gynecol Reprod Biol. 2006 Jun 1;126(2):176–9.

56. Karaki H. Historical techniques: cytosolic Ca2+ and contraction in smooth muscle. Trends Pharmacol Sci. 2004 Jul;25(7):388–93.

57. Stull JT, Hsu LC, Tansey MG, Kamm KE. Myosin light chain kinase phosphorylation in tracheal smooth muscle. J Biol Chem. 1990 Sep 25;265(27):16683–90.

58. Schwartz BG, Kloner RA. Drug Interactions With Phosphodiesterase-5 Inhibitors Used for the Treatment of Erectile Dysfunction or Pulmonary Hypertension. Circulation. 2010 Jul 6;122(1):88–95.

59. Forgue ST, Patterson BE, Bedding AW, Payne CD, Phillips DL, Wrishko RE, et al. Tadalafil pharmacokinetics in healthy subjects. Br J Clin Pharmacol. 2006 Mar;61(3):280–8.

60. Miller C, Moczydlowski E, Latorre R, Phillips M. Charybdotoxin, a protein inhibitor of single Ca2+-activated K+ channels from mammalian skeletal muscle. Nature. 1985 Jan 24;313(6000):316–8.

61. Galvez A, Gimenez-Gallego G, Reuben JP, Roy-Contancin L, Feigenbaum P, Kaczorowski GJ, et al. Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-

activated potassium channel from venom of the scorpion Buthus tamulus. J Biol Chem. 1990 Jul 5;265(19):11083–90.

62. Candia S, Garcia ML, Latorre R. Mode of action of iberiotoxin, a potent blocker of the large conductance Ca(2+)-activated K+ channel. Biophys J. 1992 Aug;63(2):583–90.

63. Dhungel KU, Kim TW, Sharma N, Bhattarai JP, Park SA, Han SK, et al. Magnesium increases iberiotoxin-sensitive large conductance calcium activated potassium currents on the basilar artery smooth muscle cells in rabbits. Neurol Res. 2012 Jan;34(1):11–6.

64. Giangiacomo KM, Garcia ML, McManus OB. Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. Biochemistry (Mosc). 1992 Jul 1;31(29):6719–27.

65. Bychkov R. Calcium-Activated Potassium Channels and Nitrate-Induced Vasodilation in Human Coronary Arteries. JPET. 1997 Dec 23;285(1):293–8.

66. Petkov GV. Central role of the BK channel in urinary bladder smooth muscle physiology and pathophysiology. Am J Physiol Regul Integr Comp Physiol. 2014 Sep 15;307(6):R571–84.

67. Wang B, Jaffe DB, Brenner R. Current understanding of iberiotoxin-resistant BK channels in the nervous system. Front Physiol [Internet]. 2014 Oct 9 [cited 2015 Sep 16];5. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4190997/

68. Shiga K, Hirano K, Nishimura J, Niiro N, Naito S, Kanaide H. Dimethyl sulphoxide relaxes rabbit detrusor muscle by decreasing the Ca2+ sensitivity of the contractile apparatus. Br J Pharmacol. 2007 Aug;151(7):1014–24.

69. Wang C-C, Lin S-Y, Lai Y-H, Liu Y-J, Hsu Y-L, Chen JJW. Dimethyl Sulfoxide Promotes the Multiple Functions of the Tumor Suppressor HLJ1 through Activator Protein-1 Activation in NSCLC Cells. PLoS ONE [Internet]. 2012 Apr 17 [cited 2015 Sep 19];7(4). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3328470/

70. Stimers JR, Song L, Rusch NJ, Rhee SW. Overexpression of the Large-Conductance, Ca2+-Activated K+ (BK) Channel Shortens Action Potential Duration in HL-1 Cardiomyocytes. PloS One. 2015;10(6):e0130588.

71. Telfer JF, Itoh H, Thomson AJ, Norman JE, Nakao K, Campa JS, et al. Activity and expression of soluble and particulate guanylate cyclases in

myometrium from nonpregnant and pregnant women: down-regulation of soluble guanylate cyclase at term. J Clin Endocrinol Metab. 2001 Dec;86(12):5934–43.

72. Bardou M, Cortijo J, Loustalot C, Taylor S, Perales-Marín A, Mercier FJ, et al. Pharmacological and biochemical study on the effects of selective phosphodiesterase inhibitors on human term myometrium. Naunyn Schmiedebergs Arch Pharmacol. 1999 Oct;360(4):457–63.

73. Pauvert O, Lugnier C, Keravis T, Marthan R, Rousseau E, Savineau JP. Effect of sildenafil on cyclic nucleotide phosphodiesterase activity, vascular tone and calcium signaling in rat pulmonary artery. Br J Pharmacol. 2003 Jun;139(3):513–22.

74. Belmonte A, Ticconi C, Dolci S, Giorgi M, Zicari A, Lenzi A, et al. Regulation of phosphodiesterase 5 expression and activity in human pregnant and non-pregnant myometrial cells by human chorionic gonadotropin. J Soc Gynecol Investig. 2005 Dec;12(8):570–7.

75. Word RA, Cornwell TL. Regulation of cGMP-induced relaxation and cGMP-dependent protein kinase in rat myometrium during pregnancy. Am J Physiol. 1998 Mar;274(3 Pt 1):C748–56.

76. Seto SW, Ho YY, Hui HN, Au ALS, Kwan YW. Contribution of glibenclamide-sensitive, ATP-dependent K+ channel activation to acetophenone analogues-mediated in vitro pulmonary artery relaxation of rat. Life Sci. 2006 Jan 2;78(6):631–9.

77. Garcia-Calvo M, Knaus HG, McManus OB, Giangiacomo KM, Kaczorowski GJ, Garcia ML. Purification and reconstitution of the high-conductance, calcium-activated potassium channel from tracheal smooth muscle. J Biol Chem. 1994 Jan 7;269(1):676–82.

78. Bolton TB. Mechanisms of action of transmitters and other substances on smooth muscle. Physiol Rev. 1979 Jul;59(3):606–718.

79. Harper MJ, Alecozay AA, Kasamo M. Prostaglandin (PG) release from human and rabbit endometrial glandular epithelial and stromal cell cultures in vitro. Adv Prostaglandin Thromboxane Leukot Res. 1991;21B:819–22.

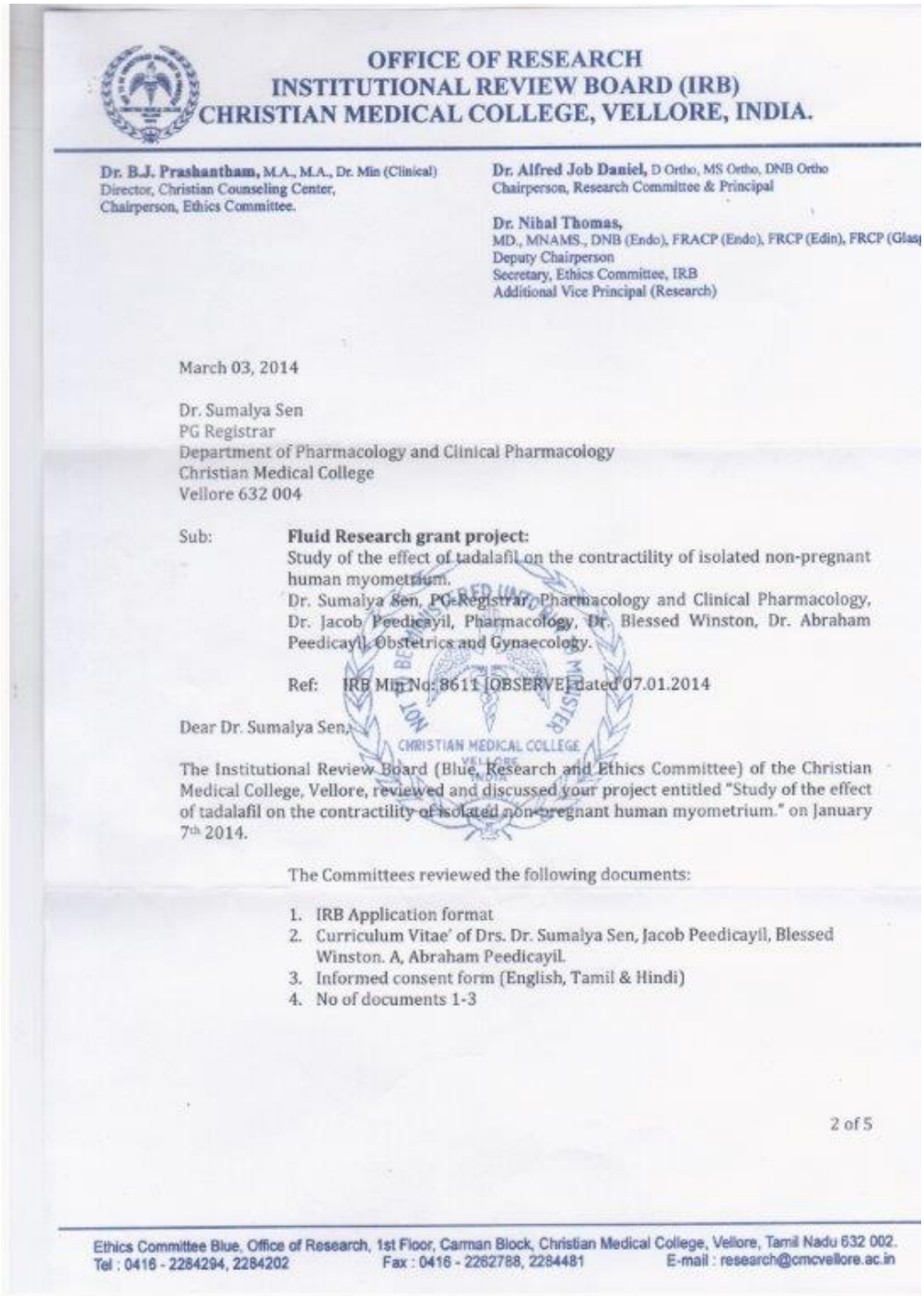
ANNEXURES

List of Figures

List of Tables

List of Tracings

Institutional review board approval letter



OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glas
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

March 03, 2014

Dr. Sumalya Sen
PG Registrar
Department of Pharmacology and Clinical Pharmacology
Christian Medical College
Vellore 632 004

Sub: **Fluid Research grant project:**
Study of the effect of tadalafil on the contractility of isolated non-pregnant human myometrium.
Dr. Sumalya Sen, PG Registrar, Pharmacology and Clinical Pharmacology, Dr. Jacob Peedicayil, Pharmacology, Dr. Blessed Winston, Dr. Abraham Peedicayil, Obstetrics and Gynaecology.

Ref: IRB Min No: 8611 [OBSERVE] dated 07.01.2014

Dear Dr. Sumalya Sen,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Study of the effect of tadalafil on the contractility of isolated non-pregnant human myometrium." on January 7th 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. Dr. Sumalya Sen, Jacob Peedicayil, Blessed Winston. A, Abraham Peedicayil.
3. Informed consent form (English, Tamil & Hindi)
4. No of documents 1-3

2 of 5

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Gla
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Jayaprakash Muliyil	B. Sc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist & Epidemiologist
Dr. Denise H. Fleming	B. Sc (Hons), PhD	Honorary Professor, Clinical Pharmacology, CMCH.	Internal, Scientist & Pharmacologist
Rev. Joseph Devaraj	B. Sc, BD	Chaplaincy Department, CMCH.	Internal, Social Scientist
Dr. Nihal Thomas,	MD, MNAMS, DNB(Endo), FRACP(Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), CMCH. Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Informed consent format

PARTICIPANT INFORMATION SHEET

Title of the study

Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium.

Name of participant _____

Hospital No._____ Date_____/_____/_____

Dear Participant,

I. You are invited to take part in this research study. The information in this document is meant to help you decide whether or not to take part in this study. This study is being conducted in the Department of Pharmacology and the Department of Obstetrics and Gynecology at Christian Medical College, Vellore.

II. Please read the instructions mentioned below carefully before signing the written informed consent document for participation in this study. Please feel free to ask if you have any queries or concerns.

III. In this study we will analyze the effects of a drug named **Tadalafil** on the uterine tissue. This study evaluates the propensity of the drug to relax the uterine muscle. If it causes relaxation, then this drug can possibly be used for the treatment of preterm labor (It is delivery of the premature baby before normal gestational period).

IV. We will collect a small strip of tissue from the uterus after its surgical removal. The tissue for the study will be taken only after adequate tissue has been sent for all the necessary investigations including biopsy, if needed.

V. You do not have to undergo any extra procedure to obtain this strip as it will be taken from the operative specimen which will be removed as a part of your current surgery.

VI. The effect of the drugs will be studied on the tissue and the specimen used will be discarded soon after each experiment. The data obtained will be stored without compromising your identity and confidentiality and the privacy of your medical information will be maintained.

VII. By signing this document, you will be allowing the research team investigators, other study personnel, Institutional Ethics Committee

and any person or agency required by law (like the Drug Controller General of India) to view your data if required.

VIII. This study may not bring you any direct benefit, however if the drug is shown to be effective in relaxing the uterine tissue it may become useful to other women suffering from preterm labor.

IX. Your participation in this research is purely voluntary. If you decide not to participate in this research study, it will in no way affect your medical care or your relationship with the treating doctor or the investigator or the institution. Also you have the right to withdraw from this study at any time of the study without giving any reason.

Thanking you for your co-operation
yours sincerely,

Dr.Sumalya Sen
Principal Investigator

Contact Persons for any queries

1) **Dr Sumalya Sen**(Principal Investigator)
Department of Pharmacology,
Christian Medical College, Vellore. Pin: 632002
Phone Number: 0416 2284237
Mobile: 09566753246
Email: sumalyasen@cmcvellore.ac.in

2) Dr.Blessed Winston (Co-Investigator)
Department of Pharmacology,
Christian Medical College, Vellore. Pin: 632002
Phone No: 0416 2284237

3) Dr. Jacob Peedicayil
Department of Pharmacology
Christian Medical College, Vellore. Pin: 632002
Phone Number: 0416-2284237
Fax: 0416-2284237
Email: jpeedi@cmcvellore.ac.in

In case of any conflicts, you can contact:

Dr. Abraham Peedicayil
Professor and Head, Department of Obstetrics and Gynaecology, Unit I
CMC Vellore, Phone Number: 04162283395

INFORMED CONSENT FORM

Title of the study

Study of the effect of Tadalafil on the contractility of isolated non-pregnant human myometrium.

Name of participant_____ Age_____

Hospital No._____ Date___/___/__ Study No._____

To whomsoever it may concern

1. I confirm that I have read and understood the participant information sheet for the above study and have had the opportunity to clarify all my doubts.[ ]
2. I have had the consent document explained to me in my native language. [ ]
3. The nature of the study has been explained to me and I state that my participation in the study is purely voluntary. I understand that I am free to withdraw at any time of the study without giving any reason, without my medical care or legal rights being affected. [ ]
4. I hereby give permission to the investigators, to use the data obtained from studying the effect of drugs on tissue obtained from the discarded uterus for publication and to release any information obtained from this study, to Government agencies, the Ethics Committee and the Regulatory authorities. I understand that they will not need my permission to look at my health records, both in respect of the current study and any further research that may be conducted in relation to it. I agree to this access. [ ]
5. I understand that the data obtained can be used in further studies without compromising my identity. [ ]
6. I understand that my identity will not be revealed in any information released to third parties or published.[ ]
7. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]
8. I have had my questions answered to my satisfaction. [ ]
9. I agree to take part in the above study. [ ]

I am aware that if I have any questions pertaining to the study at any time during this study, I can contact any one of the addresses listed as contact persons in the Participant Information Sheet.

By signing this consent form, I give my voluntary consent to participation in this study and I attest that I have understood all the information given in this document

Signature (or thumb impression) **of the Subject/Legally Acceptable Representative**

Signatory's Name _____

Date _____/_____/_____ **Time** _____AM/PM

Signature of the Impartial Witness

Witness's Name_____

Date _____/_____/_____ **Time** _____AM/PM

Signature of the Investigator

Study Investigator's Name Dr. Sumalya Sen

Date _____/_____/_____ **Time** _____AM/PM